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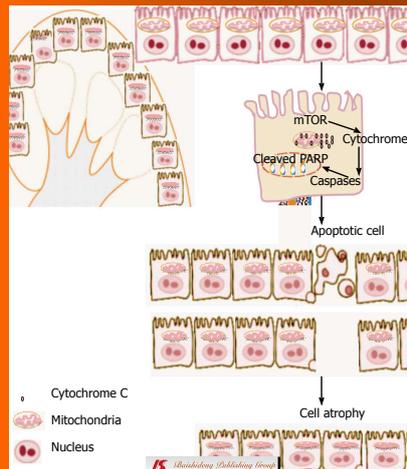


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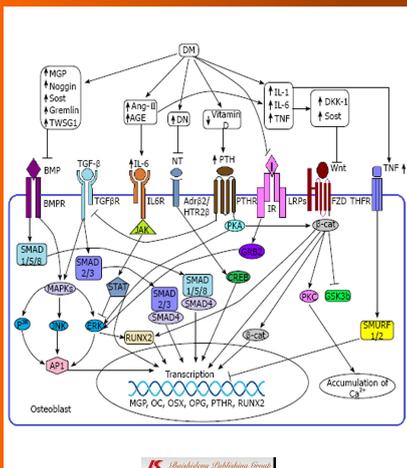


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## Contents

Bimonthly Volume 4 Number 1 February 15, 2013

### REVIEW

- 1 Insulin therapies: Current and future trends at dawn  
*Yaturu S*

### BRIEF ARTICLE

- 8 Comparison between sitagliptin and nateglinide on postprandial lipid levels:  
The STANDARD study  
*Kojima Y, Kaga H, Hayashi S, Kitazawa T, Imura Y, Ohno M, Yoshitsugu M, Fujiwara M,  
Hiyoshi T*



## Insulin therapies: Current and future trends at dawn

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### Abstract

Insulin is a key player in the control of hyperglycemia for type 1 diabetes patients and selective individuals in patients of type 2 diabetes. Insulin delivery systems that are currently available for the administration of insulin include insulin syringes, insulin infusion pumps, jet injectors and pens. The traditional and most predictable method for the administration of insulin is by subcutaneous injections. The major drawback of current forms of insulin therapy is their invasive nature. To decrease the suffering, the use of supersonic injectors, infusion pumps, sharp needles and pens has been adopted. Such invasive and intensive techniques have spurred the search for alternative, more acceptable methods for administering insulin. Several non-invasive approaches for insulin delivery are being pursued. The newer methods explored include the artificial pancreas with closed-loop system, transdermal insulin, and buccal, oral and pulmonary routes. This review focuses on the new concepts that are being explored for use in future.

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**Key words:** Diabetes; Insulin therapy; Insulin delivery systems; Oral insulin; Transdermal insulin; Inhaled insulin

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### INTRODUCTION

Insulin therapy is effective at lowering blood glucose in patients with diabetes [diabetes mellitus (DM)]. Insulin is a key player in the control of hyperglycemia for type 1 diabetes patients while it is required at later stage or in selective individuals in patients of type 2 diabetes. The discovery of insulin was considered as one of the most dramatic events in the history of the treatment of diabetes. It was isolated in 1921 with its first clinical use in 1922<sup>[1]</sup>. The major advances achieved in this area include the synthesis of human insulin analogues by recombinant technology. Insulin delivery systems that are currently available for the administration of insulin include insulin syringes, insulin infusion pumps, jet injectors and pens. The traditional and most predictable method for the administration of insulin is by subcutaneous injections. The ultimate goal would be to eliminate the need to deliver insulin exogenously and regain the ability of patients to produce and use their own insulin.

The major drawback of current forms of insulin therapy is their invasive nature. In type 1 diabetes, good glycemic control usually requires at least three or more daily insulin injections. To decrease the suffering and improve the adherence in insulin regimens, the use of supersonic injectors, infusion pumps, sharp needles and pens has been adopted. The search for more acceptable methods for administering insulin continues. Several non-invasive approaches for insulin delivery are being pursued. The success of the route of administration is measured by its ability to elicit effective and predictable lowering of blood glucose level and minimizing the risk of diabetic complications. The newer methods explored include the artificial pancreas with closed-loop system, transdermal insulin, and buccal, oral, pulmonary, nasal, ocular and rectal routes. This review focuses on the new concepts that are being explored for use in future.

## CURRENT METHODS IN INSULIN THERAPY

Use of syringes for insulin delivery is the most common method in use and it offers a wide choice of products that are easy to read and operate. Intravenous infusion of insulin was initially introduced in 1974<sup>[2,3]</sup> and low dose continuous subcutaneous infusion, in 1978<sup>[4]</sup>. Continuous subcutaneous insulin infusion (CSII), also referred to as insulin pump systems, is a way to simulate the physiology of daily insulin secretion except bypassing the liver. CSII provides a continuous supply of insulin infusion around the clock and can be individualized and can be adjusted as per the specific needs of the patient. Appropriate amounts of insulin are delivered through an infusion set. Benefits of the use of the insulin pump include avoiding change of injection sites, and providing more freedom, flexibility, and spontaneity in the person's daily life. Insulin pump therapy is very expensive as compared to the use of traditional syringes and vials. Benefits outweigh the disadvantages. Meta-analysis of CSII therapy compared to multiple daily injections in adults and adolescents with type 1 diabetes mellitus noted that CSII resulted in a greater reduction of glycated haemoglobin, in adult patients without a higher rate of hypoglycemia<sup>[5]</sup>. No beneficial effect of CSII therapy could be detected for patients with type 2 diabetes mellitus<sup>[5]</sup>.

**Insulin Pens:** Insulin pens more discreet compared with vials and syringes<sup>[6]</sup>. Insulin pens combine the insulin container and the syringe into a single modular unit. Insulin pens eliminate the inconvenience of carrying insulin vials and syringes and are more accurate and less painful. Insulin pens are user-friendly, with decreased discomfort of injection, ease of cartridge replacement, insulin-dose setting dial use and prominence of audible clicks can all affect overall dose accuracy. These are the advantages over syringes and needles<sup>[7]</sup>. Reusable insulin pens offer a wide range of advantages such as their durability, eliminating the need for cartridge refrigeration and providing flexibility in carrying a three to five day supply. Patient satisfaction and preference is higher with pen use compared to syringes and needles<sup>[8,9]</sup>.

Resistance to initiation of insulin use by many patients and some clinicians is due to concerns about its complexity or a general resistance to injections. Effective glycemic control remains an important clinical goal. Patient barriers to accepting insulin initiation with current delivery systems include fear of hypoglycemia, fear of injections, possible weight gain, and reluctance to accommodate the inflexible timing of scheduled insulin doses. Adherence issues, including dose omission, are common and are associated with some of the same factors. In addition, the invasive nature of the syringe, pump, and pen remains an obstacle for patients.

## FUTURE TRENDS

### Newer injectable insulins

Newer insulins that are promising include long acting basal insulin analogue called insulin degludec and ultra fast acting insulin, human insulin Linjeta<sup>TM</sup> (formally called VIAject).

### Insulin degludec

Insulin degludec, a novel ultra-long acting basal insulin, is almost identical to human insulin in structure except for the last aminoacid deleted from the B-chain and addition of a glutamyl link from LysB29 to a hexadecandioic fatty acid<sup>[10]</sup>. This insulin forms soluble multihexamers after subcutaneous injection, resulting in an ultra-long action profile with half life more than 24 h.

Insulin degludec has proven to be non inferior to insulin glargine in clinical trials carried out in both type 1 and type 2 DM. Exploratory studies in type 1 diabetes have shown insulin degludec to be safe with reduced rates of hypoglycemia and comparable glycemic control to long acting insulin analogue insulin glargine<sup>[11]</sup>. Phase 3 clinical trials in adults with type 1 DM<sup>[12]</sup> and type 2 DM glycemic controls was comparable to insulin glargine at one year follow up with fewer hypoglycemic episodes. As insulin degludec has an ultra-long acting profile, insulin degludec was studied using injections three times a week compared with insulin glargine once a day and found to have comparable response<sup>[13]</sup>. The advantages of insulin degludec were reviewed in several recent publications<sup>[14-16]</sup>. Comparative studies of efficacy and safety of insulin degludec and insulin glargine, both administered once daily with mealtime insulin aspart, in basal-bolus therapy for type 1 diabetes<sup>[12]</sup> and type 2 diabetes<sup>[17]</sup> noted effective glycaemic control with a lower risk of nocturnal hypoglycemia than insulin glargine. Similar studies comparing insulin degludec along with aspart insulin compared to insulin detemir with aspart insulin noted improved overall glycemic control while lowering the risk of nocturnal hypoglycemia and fewer injections<sup>[18]</sup>. Insulin degludec is not yet approved by Food and Drug Administration.

**VIAject<sup>TM</sup>:** VIAject is a recombinant human insulin with ultra fast onset of action. Pharmacodynamic and pharmacokinetic studies have shown the onset of action of VIAject is faster than that of human soluble insulin and insulin lispro<sup>[19]</sup>. VIAject was reported to have less within-subject variability of plasma insulin compared to human regular insulin<sup>[20]</sup>, and has a faster absorption/onset of action than insulin lispro<sup>[21,22]</sup>. Two pivotal phase III clinical studies in both type 1 and type 2 DM are ongoing with VIAject. As the amount of insulin circulating several hours after a meal is low, a possible reduction in hypoglycemia and prevention of weight gain are predicted.

## ARTIFICIAL PANCREAS

Introduction of continuous glucose sensors<sup>[23]</sup> has led

to development of the artificial pancreas, which made improved care possible. Even with the use of continuous glucose monitors and insulin pumps, most people with type 1 DM do not achieve glycemic goals and continue to have unacceptable rates of hypoglycemia. Closed-loop insulin delivery, also referred to as the artificial pancreas, is an emerging therapeutic approach for people with type 1 DM. In this closed-loop, blood glucose control is achieved using an algorithm, wireless communication of a continuous glucose monitor linked to insulin infusion pump that facilitates automated data transfer and delivers insulin, without the need for human intervention. The goal of closed-loop therapy is to achieve good glycemic control with the use of a control algorithm that directs insulin delivery according to glucose levels while reducing the risk of hypoglycemia.

Beta cells respond to circulating glucose levels by feedback mechanism. Insulin delivery in the closed loop system is modulated at intervals of 1 to 15 min, depending on interstitial glucose levels. The novelty of this approach resides in the real-time feedback between glucose levels and insulin delivery, similar to that of the beta-cell. The algorithms that are most relevant of the available various algorithms include the proportional-integral-derivative control and the model-predictive control. True closed-loop systems, that determine minute-to minute insulin delivery based on continuous glucose sensor data in real-time, have shown promise in small inpatient feasibility studies, using a variety of algorithmic and hormonal approaches<sup>[24]</sup>. To have a near normal closed-loop system, several areas need to be improved. First and foremost is the rapid onset of action. Lag period of current fast-acting insulin analogs is 90-120 min. The limitations of current glucose sensors include a lag period, as they measure interstitial fluid rather than blood glucose, and errors from transient loss of sensitivity<sup>[24]</sup>. Rapid acting insulins are being developed. Addition of recombinant human hyaluronidase (rHuPH20) accelerates insulin absorption. Current trials show promise. Both lispro and recombinant human insulin with rHuPH20 in phase 2 studies noted earlier and greater peak insulin concentrations and improved postprandial glycemic control and reduced hypoglycemia<sup>[25]</sup>. Use of monomeric insulins that cannot form hexamers are being developed<sup>[26]</sup>. As mentioned earlier, ultrafast insulin VIAject, a formulation of human soluble insulin improves the rate of insulin absorption. Steiner and associates have reported that VIAject has higher metabolic activity in the first 2 h after injection as noted in their study to evaluate the pharmacodynamic and pharmacokinetic properties<sup>[19]</sup>.

## BUCCAL DELIVERY OF INSULIN

Transmucosal delivery is a suitable route for insulin non-injection administration. Insulin delivered by buccal delivery system is through an aerosol spray into the oral cavity and hence, differs from inhalers. The insulin is absorbed through the inside of the cheeks and in the back of the

mouth instead of the lungs. Nanoparticles are pelleted to impart three-dimensional structural conformity and coherence thereby facilitating of buccal delivery of insulin. *In vivo* studies performed on diabetic rats showed promising results with stable blood glucose profile with a significant hypoglycemic response after 7 h<sup>[27]</sup>. Similar studies in the rabbit and rat have shown that buccal spray of insulin is an effective insulin delivery system, which is promising for clinical trial and future clinical application<sup>[28]</sup>. Though results are promising in rat models, rats are not appropriate models as rats have a keratinized buccal mucosa. The only animal models comparable to the human buccal permeability are pigs. The continuous, but variable, saliva flow and the robust multilayered structure of the oral epithelium constitute another effective barrier to penetration of drugs. Oral-Lyn, Generex Biotechnology Corporation, Toronto, Canada is developing a buccal insulin formulation, based on RapidMist, advanced buccal drug delivery technology<sup>[29]</sup> ([www.Generex.com/technology.php](http://www.Generex.com/technology.php)). Oral-lyn is a liquid formulation of human regular insulin with a spray propellant for prandial insulin therapy. The insulin formulation is said to be stable at room temperature for more than six months. The formulation results in an aerosol with relatively large micelles (85% of that having a mean size > 10  $\mu\text{m}$ ) and therefore cannot go into the lungs. Each puff is claimed to deliver 10 U of insulin. Absorption rate of insulin administered as a puff is 10% and that corresponds to 1 U when one puff of 10 U is delivered. That translates to use of 10 puffs to deliver 10 U insulin for a meal; this undertaking can be considered time-consuming and not user friendly. The insulin is claimed to be released from the device as a metered dose, identical from first puff to the last<sup>[29]</sup>.

Clinical studies in healthy volunteers and subjects with type 1 DM and type 2 DM have shown that the oral insulin spray was absorbed in direct relation to the amount given and had a faster onset and a shorter duration of action when compared with regular insulin given subcutaneously. In all of the studies conducted, the oral insulin spray was generally well tolerated. Only side effects noted include mild, self-limited episodes of transient (1-2 min) mild dizziness during dosing in some healthy volunteers and subjects with type 1 DM. No changes in vital signs, laboratory values or physical examination results were said to have occurred<sup>[30]</sup>. The product is said to be on the market in a number of countries (*e.g.*, Ecuador and India)<sup>[29]</sup>. Without appropriately designed and performed phase II and III trials at hand, it is not possible to make any clear statement about the benefits/risk ratio of the different buccal insulin<sup>[29]</sup>. Some companies are quite active and a small Israel-based company Oramed is in phase 2b<sup>[29]</sup>.

## ORAL INSULIN

Since the initial discovery of insulin by Banting and Best in 1922, an oral form of insulin was the elusive goal. Oral insulin has benefits in terms of fostering compliance and adherence among patients, as well as physiologic advan-

tages due to the fact that oral insulin can mimic the physiological fate of insulin through the portal vein and target the liver directly and inhibit the hepatic glucose production<sup>[31]</sup>. Insulin being a protein, difficulties encountered in oral delivery include degradation by low pH of the stomach and different digestive enzymes in the stomach and small intestine; and the major barrier for absorption is the intestinal epithelium. All these lead to low bioavailability and that leads to significant inter- and intra-subject variability.

Nano technology has brought some hope. Nano particles composed of naturally occurring biodegradable polymers have emerged as potential carriers of various therapeutic agents for controlled drug delivery through the oral route. Nanotechnology application to delivery of hydrophilic drugs such as insulin is still a challenge, and includes prodrugs (insulin-polymer conjugation), micelles, liposomes, solid lipid nano particles (NPs) and NPs of biodegradable polymers. Chitosan, a cationic polysaccharide, is one of such biodegradable polymers, which has been extensively exploited for the preparation of nano particles for oral controlled delivery of several therapeutic agents<sup>[32-36]</sup>. The area of focus has shifted from chitosan to chitosan derivatized polymers that improve drug retention capability, and provide improved permeation, enhanced mucoadhesion and sustained release of therapeutic agents<sup>[37,38]</sup>.

The newer products that are being tried include water-soluble, long-acting insulin derivative, [(2-Sulfo)-9-fluorenylmethoxycarbonyl]3-insulin<sup>[39]</sup>, vitamin B12-dextran nano particles<sup>[40]</sup>, lipid nano particles<sup>[41]</sup> and PEGylated calcium phosphate nano particles as oral carriers for insulin<sup>[42]</sup>. Protection of insulin from the gastric environment has been achieved by coating the nano particles with a pH sensitive polymer that will dissolve in the mildly alkaline pH environment of the intestine. A sustained release of insulin was observed at neutral (intestinal) pH for over 8 h and it was concluded that PEGylated calcium phosphate nano particles are an excellent carrier system for insulin<sup>[42]</sup>. So far the studies are in animals, both in normal and diabetic rats, respectively<sup>[43]</sup>. Biocon company that is manufacturing IN-105 seems to be aggressively working on development of oral insulin, IN-105 and is in late phase 3 clinical trials<sup>[29]</sup>.

### IN-105

Oral insulin IN-105 is an insulin analog. It is a second-generation of oral insulin that has an attractive stability profile at ambient conditions. It is a human recombinant insulin molecule conjugated on position B29 with polyethylene glycol *via* an acetyl chain. IN-105 is said to have improved half-life in the digestive tract and improved absorption, lower immunogenicity as compared to insulin. It said to have lower mitogenic potential as compared to insulin but retains a similar pharmacological activity as insulin, and conserves the safety profile and good clearance profile as compared to insulin. Extensive preclinical studies in different species have shown no issues in acute

dose toxicity studies. Studies to address genotoxicity, mutagenicity, reproductive toxicity and teratogenicity have shown nothing. Maximal circulating insulin levels after oral administration of 5 mg IN-105 were observed after 20 min with maximum drop in glucose at 40 min. However, the rapid decline in blood glucose might have induced a counter regulatory response that induces an increase in glycemia *per se*<sup>[29]</sup>. Phase 1 and phase 2 trials were promising. In a dose escalating study, IN-105 absorption was shown to be proportional to the dose administered. The 2-h postprandial glucose excursion was also said to have reduced in a dose proportional manner<sup>[44]</sup>.

## INHALED INSULIN

The lung provides an attractive and ideal route on account of its accessibility and its large surface area and large alveolar-capillary network for drug absorption. Insulin inhalers would work much like asthma inhalers. The products fall into two main groups: the dry powder formulations and solution, which are delivered through different patented inhaler systems. Exubera<sup>®</sup>, containing rapid-acting insulin in powder form, has been studied extensively in patients with type 1 and type 2 diabetes mellitus<sup>[45,46]</sup>. A patient preference study, using a comparison of utility scores, showed that a majority prefers the inhaled route and the minority prefers the injectable route<sup>[47]</sup>. However, issues like cost, bulky device, fear for lung safety, and the small number of studies in subjects with underlying respiratory disease prevented widespread use of this new mode of delivery<sup>[48,49]</sup>. Exubera<sup>®</sup>, was available for a short time (August 2006 to October 2007). In October 2007, Pfizer took off Exubera off the market as the drug failed to gain market acceptance.

### Afrezza

Afrezza is recombinant human insulin, using the technosphere concept and administered using MannKind's next-generation inhaler called Dreamboat. Technosphere is a drug delivery system created by micro particles (2-3  $\mu\text{m}$ ), which form microspheres, which are then lyophilized into a dry powder for inhalation<sup>[50]</sup>. Technosphere insulin is an inhaled form of regular human insulin with a rapid onset of action (about 15 min) that is being considered for approval for the treatment of type 1 and type 2 DM and is currently in phase 3 clinical trials.

Most of the published evidence regarding Technosphere insulin's efficacy has been in patients with type 2 DM. The observed changes in lung function with Technosphere insulin were reported to be small and said to have occurred within the first 3 mo of therapy that remained non-progressive over 2 years<sup>[51]</sup>. In comparison with insulin aspart, in a phase 3 randomized controlled trial, meal-time Technosphere insulin plus insulin glargine was found to be noninferior<sup>[52]</sup>. Technosphere insulin was reported to be well tolerated by the patients in clinical trials. Rates of hypoglycemia and weight gain were similar to other insulin regimens. The most commonly reported signifi-

cant side effect was an increase in the frequency of cough reported. Since Exubera, the previously marketed inhaled insulin, mentioned a potential link to lung cancer in its product labeling, even though causation had not been established, long term studies with Technosphere insulin were requested by the Food and Drug Administration to detect potential additional harms, such as lung cancer.

### Transdermal insulin

Transdermal insulin delivery is an attractive needle-free alternative and avoids the disadvantages associated with the invasive parenteral route of administration and other alternative routes such as the pulmonary and nasal routes. Permeation of compounds is limited to small, lipophilic molecules, as the stratum corneum, the outermost layer of the skin constitutes the major barrier. Several chemical and physical enhancement techniques, such as iontophoresis, ultrasound/sonophoresis, microneedles, electroporation, laser ablation and chemical enhancers, have been explored to overcome the stratum corneum barrier to increase skin permeability. The advantages of transdermal drug delivery include convenience, good patient compliance, prolonged therapy, and avoidance of both the liver's first-pass metabolism and degradation in the gastrointestinal tract. To improve transdermal delivery, microneedles have been regarded as a potential technology approach to be employed alone or with other enhancing methods such as electroporation and iontophoresis, as well as with different drug carriers (*e.g.*, lipid vesicles, micro- and nanoparticles)<sup>[53]</sup>. As microneedles inserted into the skin of human subjects are reported to be painless, microneedles are a promising technology to deliver drugs into the skin<sup>[54]</sup>.

### Methods to improve transdermal delivery

Chemical enhancers alter the lipid structure of the stratum corneum thereby reducing its barrier properties and increasing its permeability for drugs which would not pass through the skin passively. Iontophoresis is a technique that enhance the transdermal delivery of compounds through the skin *via* the application of a small electric current<sup>[55]</sup>. Microneedle technology offers a cost-effective, minimally invasive, and controllable approach to transdermal drug delivery. It involves the creation of micron-sized channels in the skin, thereby disrupting the stratum corneum barrier. Upon creation of the microchannels, interstitial fluid fills up the channels, resulting in hydrophilic pathways<sup>[56]</sup>. Microneedles deliver the drug into the epidermis without disruption of nerve endings<sup>[57]</sup>. Sonophoresis (phonophoresis) uses ultrasound and it has been shown to increase skin permeability to various low and high molecular weight drugs, including insulin. However, its therapeutic value is still being evaluated<sup>[58]</sup>. Microdermabrasion is a method to increase skin permeability for transdermal drug delivery by damaging or removing skin's outer layer, stratum corneum<sup>[59]</sup>. Microdermabrasion can increase skin permeability to deliver insulin<sup>[60]</sup>.

Patches deliver basal insulin rather than a fast-acting

bolus, hence are not useful for meal time boluses. Preliminary data on insulin-loaded micro-emulsions for transdermal delivery showed promise on goat skin<sup>[61]</sup>. Altea Development Corporation is planning to introduce a product which will either be a one- or half-day patch, depending on the outcome of testing.

## CONCLUSION

Recent developments in insulin therapy have potential for reducing some of the negative aspects of current methods. Long-acting insulin, such as insulin degludec, may require less frequent injections. Fast-acting insulin, such as Viaject, have been shown to improve postprandial glycemic control and reduce hypoglycemia. The artificial pancreas (closed-loop systems with insulin pumps that deliver insulin in response to sensors) may prove to be a valuable therapy for type 1 diabetes patients, particularly if the lag period can be shortened through improved glucose sensors and the use of ultra-fast acting insulin. Of the alternative methods of administration, the oral route is the most promising, especially with nanotechnology allowing for several types of encapsulations to bypass the gastric acidic environment. Oral delivery offers the benefits of ease of administration (leading to greater acceptance by patients), improved absorption rates, and mimicry of the normal route of insulin through the liver.

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## Comparison between sitagliptin and nateglinide on postprandial lipid levels: The STANDARD study

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### Abstract

**AIM:** To assess the effects of sitagliptin and nateglinide on lipid metabolism.

**METHODS:** In a parallel group comparative open trial, patients with type 2 diabetes mellitus under treatment at the Japanese Red Cross Medical Center were randomly assigned to receive either sitagliptin (50 mg once daily) or nateglinide (90 mg three times daily before meals). Eligible patients met the following criteria: age  $\geq 20$  years; hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>)  $> 6.5\%$  despite diet and exercise; HbA<sub>1c</sub> between 6.5% and 8.0%; fasting glucose  $< 7.77$  mmol/L; diet and exercise therapy for more than 3 mo; and ability to read and understand the information for written informed consent. Exclusion criteria were contraindications to sitagliptin, contraindications to nateglinide, pregnancy or possible pregnancy, and severe liver/renal failure. Patients who were considered to be unsuitable by the attending physician for other reasons were also excluded. Blood

samples were collected at one and three hours after intake of a test meal. The primary outcome measure was the area under the curve (AUC) of apolipoprotein (Apo) B48 at three hours postprandially.

**RESULTS:** Twenty patients were randomly assigned to the sitagliptin group and sixteen patients were randomized to the nateglinide group. All 36 patients took the medication as directed by the physician in both groups, and they all were analyzed. Apart from anti-diabetic drugs, there was no difference between the two groups with respect to the frequency of combined use of lipid-lowering, antihypertensive, and/or anti-platelet drugs. The doses of these medications were maintained during 12 wk of treatment. Detailed dietary advice, together with adequate exercise therapy, was given to the patients so that other factors apart from the two test drugs were similar in the two groups. There were no significant differences of the baseline characteristics between the two groups, except for body mass index (the sitagliptin group:  $25.14 \pm 3.05$  kg/m<sup>2</sup>; the nateglinide group:  $21.39 \pm 2.24$  kg/m<sup>2</sup>). Fasting levels of HbA<sub>1c</sub>, glycated albumin, 1,5-anhydroglucitol, and blood glucose, as well as the blood glucose levels at one and three hours postprandially, improved in both groups after 12 wk of treatment, and there were no significant differences between the two groups. However, the glucagon level at one hour postprandially ( $P = 0.040$ ) and the diastolic blood pressure ( $P < 0.01$ ) only showed a significant decrease in the sitagliptin group. In the nateglinide group, there was no significant change in the AUC of Apo B48, the glucagon level at one hour postprandially, the fasting triglyceride level, or the diastolic blood pressure. Body weight was unchanged in both groups. However, the AUC of Apo B48 at three hours postprandially showed a significant decrease in the sitagliptin group from  $2.48 \pm 0.11$  at baseline to  $1.94 \pm 0.78$  g/L per hour after 12 wk ( $P = 0.019$ ). The fasting triglyceride level also decreased significantly in the sitagliptin group ( $P$

= 0.035). With regard to lipid-related markers other than Apo B48 and fasting triglycerides, no significant changes were observed with respect to Apo A1, Apo B, or Apo C3 in either group. No adverse events occurred in either group.

**CONCLUSION:** Sitagliptin significantly improves some lipid parameters while having a comparable effect on blood glucose to nateglinide. A large-scale prospective study of sitagliptin therapy is warranted.

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**Key words:** Dipeptidyl-peptidase 4 inhibitors; Type 2 diabetes mellitus; Sitagliptin; Nateglinide; Blood glucose; Lipid metabolism

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## INTRODUCTION

When treating diabetes mellitus, prevention of major vascular disorders is an important objective<sup>[1-9]</sup>. Several studies have demonstrated that postprandial hyperglycemia (PPHG) is significantly linked to major vascular disorders<sup>[10-16]</sup>. In an *in vitro* study of human vascular endothelial cells, the cellular death rate was increased by repeated fluctuation between normoglycemia and hyperglycemia compared with that due to persistent hyperglycemia<sup>[17]</sup>. These findings suggest that inhibition of circadian glycaemic changes may prevent vascular disorders<sup>[18,19]</sup>.

Postprandial hyperlipidemia (PPHL) has been noted in patients with type 2 diabetes. Inhibition of lipoprotein lipase activity due to impaired insulin action on adipocytes and an increase of exogenous lipoproteins due to overeating are considered to be the underlying causes of PPHL. PPHL may trigger progression of arteriosclerosis with the accumulation of chylomicron remnants in the vessel walls. An epidemiological study of Japanese patients has shown that hypertriglyceridemia is an independent risk factor for coronary artery disorders<sup>[20]</sup>.

Nateglinide, a *D*-phenylalanine derivative, rapidly stimulates insulin secretion to exert an antihyperglycemic effect and treatment with nateglinide inhibits carotid artery intima-media thickening, which is a surrogate marker of arteriosclerosis<sup>[21]</sup>. On the other hand, the dipeptidyl peptidase-4 (DPP-4) inhibitor sitagliptin increases insulin secretion by inhibiting breakdown of incretins, thereby exerting an antihyperglycemic effect. The efficacy of sitagliptin for PPHL has also been reported<sup>[22,23]</sup>. However, it is unknown whether this improvement of PPHL is due to suppression of postprandial glycemia or an increase of incretin secretion secondary to inhibition of DPP-4.

In the present study, we treated type 2 diabetic patients with nateglinide or sitagliptin for 12 wk. The response to a test meal was assessed to compare the effects of the two drugs on PPHG and PPHL.

## MATERIALS AND METHODS

### Study population

Patients with type 2 diabetes attending the Japanese Red Cross Medical Center (JRCMC) between July 2010 and June 2011 were enrolled in this parallel group comparative open trial. Eligible patients met the following criteria: age  $\geq$  20 years; hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>)  $>$  6.5% despite diet and exercise; HbA<sub>1c</sub> between 6.5% and 8.0%; fasting glucose  $<$  7.77 mmol/L; diet and exercise therapy for more than 3 mo; and ability to read and understand the information for written informed consent. Exclusion criteria were contraindications to sitagliptin, contraindications to nateglinide, pregnancy or possible pregnancy, and severe liver/renal failure. We also excluded patients who were considered to be unsuitable by the attending physician for other reasons. As many patients as possible were registered because it was difficult to set a specific sample size for this exploratory clinical study and relevant information was limited.

### Randomization

Random allocation of patients to either treatment group was performed centrally by using our registration system, with the random allocation sequence being generated by Kojima Y. Enrollment was done by Kojima Y, Kaga H and Kitazawa T, while assignment of participants to interventions was carried out by Iimura Y, Ohno M, Yoshitsugu M and Hiyoshi T.

### Study treatment

Sitagliptin was administered at a dose of 50 mg once daily before breakfast to one group, while nateglinide was administered at a dose of 90 mg three times daily (immediately before each meal) to the other group. Doses of antihypertensive and lipid-lowering drugs were not changed during the study.

### Data collection

Blood samples were collected before and one and three hours after eating a test meal (total energy: 1925 kJ; protein: 18.0 g, fat: 18.0 g, carbohydrate: 56.5 g), both before treatment and after the 12-wk treatment period (final date: September 30, 2011). Then the levels of various parameters of glucose and lipid metabolism were measured.

### Outcome measures

The primary outcome was the area under the concentration *vs* time curve (AUC) of apolipoprotein (Apo) B48 at three hours postprandially after 12 wk of treatment. The secondary outcomes were postprandial blood glucose, HbA<sub>1c</sub>, glucagon at one hour postprandially, fasting triglycerides, and blood pressure.

**Table 1** Baseline patient profile and post-treatment data

	Sitagliptin group		Nateglinide group	
	Before treatment	After 3 mo of treatment	Before treatment	After 3 mo of treatment
Age (yr)	63.85 ± 12.92	-	66.44 ± 9.02	-
Sex: M/F (n)	15/5	15/5	12/4	12/4
Body weight (kg)	68.79 ± 12.89 <sup>1</sup>	69.30 ± 13.18	58.36 ± 8.54	58.75 ± 8.52
Systolic blood pressure (mmHg)	134.3 ± 19.3	126.5 ± 11.1	129.1 ± 13.9	122.7 ± 14.8
Diastolic blood pressure (mmHg)	81.5 ± 12.9	73.3 ± 7.4 <sup>b</sup>	76.9 ± 8.8	74.3 ± 10.2
HbA <sub>1c</sub> (%)	7.2 ± 0.7	7.0 ± 0.8 <sup>b</sup>	7.2 ± 0.4	6.8 ± 0.5 <sup>b</sup>
Glycated albumin (%)	19.3 ± 3.6	17.7 ± 2.6 <sup>b</sup>	19.9 ± 3.4	18.1 ± 2.8 <sup>b</sup>
1.5-AG (μmol/L)	65.5 ± 45.8	81.6 ± 46.2 <sup>b</sup>	51.0 ± 22.7	69.5 ± 29.3 <sup>b</sup>
Fasting blood glucose (mmol/L)	7.8 ± 1.7	7.3 ± 1.6	8.1 ± 1.0	7.6 ± 1.5
Blood glucose 1 h postprandially (mmol/L)	12.0 ± 3.0	10.6 ± 3.1 <sup>b</sup>	13.6 ± 2.3	11.0 ± 2.8 <sup>b</sup>
Blood glucose 3 h postprandially (mmol/L)	7.6 ± 2.5	6.6 ± 2.0 <sup>b</sup>	7.6 ± 2.5	6.5 ± 1.9 <sup>a</sup>
Apo-B48 AUC (g/L per hour)	2.48 ± 0.11	1.94 ± 0.78 <sup>a</sup>	3.14 ± 0.29	2.29 ± 0.16
Apo-A1 AUC (g/L per hour)	4.64 ± 0.73	4.50 ± 0.76	4.59 ± 0.73	4.67 ± 0.76
Apo-B AUC (g/L per hour)	2.73 ± 0.47	2.91 ± 0.46	2.60 ± 0.46	2.64 ± 0.40
Apo-C3 AUC (g/L per hour)	0.30 ± 0.07	0.28 ± 0.06	0.31 ± 0.11	0.28 ± 0.09
Fasting insulin (pmol/L)	53.3 ± 26.9	58.4 ± 34.4	41.3 ± 29.0	40.4 ± 31.5
Insulin 1 h postprandially (μU/mL)	279.3 ± 115.7	275.1 ± 143.4	230.1 ± 104.2	248.7 ± 112.2
Fasting glucagon (ng/L)	67.2 ± 15.9	66.6 ± 16.0	65.1 ± 16.4	70.1 ± 16.0
Glucagon 1 h postprandially (ng/L)	80.3 ± 17.0	70.3 ± 16.9 <sup>a</sup>	79.0 ± 13.4	81.5 ± 20.6
Fasting triglycerides (mmol/L)	1.26 ± 0.81	1.09 ± 0.61 <sup>a</sup>	1.54 ± 1.44	1.11 ± 0.60

<sup>1</sup>This parameter showed a significant between-group difference ( $P < 0.05$ ) before treatment. Data are shown as the mean ± SD (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs time curve). Area under the concentration vs time curve (AUC) data were obtained from before to three hours after the test meal. The *t* test was used for statistical analysis. M: Male; F: Female; 1.5-AG: 1.5-anhydroglucitol; Apo: Apolipoprotein; HbA<sub>1c</sub>: Hemoglobin A<sub>1c</sub>.

### Ethical considerations

This study was approved by the Ethical Committee of the JRCMC and was conducted according to the principles specified in the Helsinki Declaration. Prior to its initiation, we registered this study with the UMIN Clinical Trials Registry (www.umin.ac.jp; registration number UMIN000006278), as the sitagliptin and nateglinide randomized clinical trial (STANDARD study). All patients provided informed consent and participated in the study on a voluntary basis.

### Statistical analysis

For intragroup and intergroup comparisons, we employed the paired *t* test and *t* test, respectively. All analysis were carried out by using SAS software version 8.02 (SAS Institute, Cary, NC, United States), and  $P < 0.05$  was considered statistically significant.

## RESULTS

Twenty patients were randomly assigned to the sitagliptin group and sixteen patients were randomized to the nateglinide group. All 36 patients took the medication every day as directed by the physician in both groups, and they all were available for analysis. Apart from antidiabetic drugs, there was no difference between the two groups with respect to the frequency of combined use of lipid-lowering, antihypertensive, and/or antiplatelet drugs (statins in 6 and 7 patients from the sitagliptin group and the nateglinide group, respectively; angiotensin receptor blockers in 6 and 3 patients; and calcium channel blockers in 1 and 2 patients). The doses of these medications were maintained during 12 wk of treatment. Detailed dietary

advice, together with adequate exercise therapy, was given to the patients by diabetologists, nurses, and dieticians who belonged to the JRCMC, so that other factors apart from the two test drugs were similar in the two groups.

Table 1 summarizes the baseline and post-treatment data. There were no significant differences of the mean age, sex ratio, HbA<sub>1c</sub>, 1.5-anhydroglucitol (1.5-AG), glycated albumin, Apo A1, Apo B, Apo B48, Apo C3, fasting triglycerides, postprandial blood glucose, insulin, and glucagon levels. However, the baseline body mass index was higher in the sitagliptin group than in the nateglinide group ( $25.14 \pm 3.05$  kg/m<sup>2</sup> vs  $21.39 \pm 2.24$  kg/m<sup>2</sup>).

Fasting levels of HbA<sub>1c</sub>, glycated albumin, 1.5-AG and blood glucose, as well as the blood glucose levels at one and three hours postprandially, improved in both groups after 12 wk of treatment, and there were no significant differences between the two groups. However, the glucagon level at one hour postprandially ( $P = 0.040$ ) and the diastolic blood pressure ( $P < 0.01$ ) only showed a significant decrease in the sitagliptin group. In the nateglinide group, there was no significant change in the AUC of Apo B48, the glucagon level at one hour postprandially, the fasting triglyceride level, or the diastolic blood pressure. Body weight was unchanged in both groups. However, the AUC of Apo B48 at three hours postprandially showed a significant decrease in the sitagliptin group from  $2.48 \pm 0.11$  g/L at baseline to  $1.94 \pm 0.78$  g/L per hour after 12 wk ( $P = 0.019$ ). The fasting triglyceride level also decreased significantly in the sitagliptin group ( $P = 0.035$ ). With regard to lipid-related markers other than Apo B48 and fasting triglycerides, no significant changes were observed with respect to Apo A1, Apo B, or Apo C3 in either group.

No adverse events, including hypoglycemia, gastrointestinal side effects, pancreatitis, infection, immune abnormalities, liver dysfunction, renal dysfunction, and cardiac dysfunction, occurred in either group.

## DISCUSSION

The present study showed that two oral antidiabetic drugs with different mechanisms of action similarly improved postprandial glycemia and significantly decreased the levels of HbA<sub>1c</sub>, glycated albumin, and 1.5-AG. With regard to lipid metabolism, however, a significant decrease of fasting triglycerides and of the postprandial AUC for Apo B48, a marker of chylomicrons and chylomicron remnants (exogenous lipids), only occurred in the sitagliptin group. There was no change of the AUC for Apo B (endogenous lipids) in either group.

Tremblay *et al.*<sup>23</sup> investigated postprandial changes of lipoproteins after administration of sitagliptin for 6 wk and reported a significant decrease of triglyceride-rich lipoproteins (both Apo B and Apo B48), suggesting that sitagliptin reduced the levels of endogenous and exogenous lipids. In contrast, our study showed that Apo B did not decrease after 12 wk of treatment with sitagliptin, while Apo B48 decreased significantly. This difference may be explained by different conditions of the two studies, including the lipid and calorie contents of the test meals. Apo C3 is related to inhibition of lipoprotein lipase activity in hepatic cells, and it showed no significant change in either group, which suggests that the Apo C3-related pathway for synthesis of endogenous lipids was not affected by either medication. In the sitagliptin group, it is thought that inhibition of gastrointestinal peristalsis leads to reduced absorption of triglycerides and decreased Apo B48 synthesis.

Lee *et al.*<sup>24</sup> investigated the serum glucagon levels of healthy individuals and patients with impaired glucose tolerance or type 2 diabetes and reported that the differences between these groups were maximal at one hour postprandially. In the present study, a significant decrease of the serum glucagon level at one hour postprandially was only noted in the sitagliptin group, suggesting that sitagliptin and nateglinide have differing effects on glucagon. In addition, the influence of glucagon on postprandial lipid levels requires further investigation.

Nateglinide has been reported to improve PPHG through its insulinotropic action, and nateglinide treatment caused no appreciable changes of serum lipid levels in this study. The emphasis of treatment for type 2 diabetes has shifted from simple correction of fasting and PPHG to management of dyslipidemia as well. Because sitagliptin inhibits both PPHG and PPHL, it may be useful for the prevention of macrovascular disease in patients with diabetes<sup>[25-29]</sup>, but a large-scale prospective study would be required for confirmation.

This study had the following limitations: (1) small sample sizes (20 and 16 per group, respectively); (2) unequal baseline body weights of the two groups; and (3) a

short follow-up period of 3 mo. Accordingly, our results have limited generalizability and a large-scale long-term study is needed to verify our findings.

In conclusion, our randomized controlled trial showed that suppression of triglycerides and postprandial Apo B48 only occurred in the sitagliptin group, although PPHG was similarly improved in both the sitagliptin and nateglinide groups. To best of our knowledge, this is the first report to confirm the efficacy of a DPP-4 inhibitor for improving postprandial hyperlipidemia while achieving a comparable improvement of blood glucose to the control drug. A large-scale prospective study of sitagliptin therapy is needed.

## COMMENTS

### Background

There have been some reports about the efficacy of sitagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, for postprandial hyperlipidemia in patients with type 2 diabetes. However, it is unknown whether this improvement of postprandial hyperlipidemia is due to suppression of postprandial glycemia or an increase of incretin secretion secondary to inhibition of DPP-4.

### Research frontiers

Nateglinide, a *D*-phenylalanine derivative, rapidly stimulates insulin secretion to exert its antihyperglycemic effect. Sitagliptin was the first incretin enhancer approved in Japan (in 2009) and it increases insulin secretion by inhibiting the breakdown of incretins, unlike nateglinide. It remains unclear which of these two drugs is more effective for improving postprandial hyperlipidemia and hyperglycemia.

### Innovations and breakthroughs

This is the first study to demonstrate that sitagliptin significantly improves some lipid parameters while having a comparable effect on blood glucose to nateglinide, with a decrease of triglycerides and apolipoprotein (Apo) B48 only being observed in patients receiving sitagliptin.

### Applications

By understanding and utilizing the response to sitagliptin demonstrated in this study, treatment of diabetes could be better tailored and macrovascular disease may also be prevented because sitagliptin inhibits both postprandial hyperglycemia and postprandial hyperlipidemia.

### Terminology

DPP-4 inhibitors, of which sitagliptin was the first to be released in Japan, inhibit the enzyme DPP-4 and are used to treat type 2 diabetes. Apo B is the major component of the apolipoproteins, which serve as enzyme cofactors, receptor ligands, and lipid carriers that regulate the metabolism of lipoproteins and their tissue uptake.

### Peer review

In this 12-wk trial, the authors tested the effects of sitagliptin and nateglinide on metabolic markers in 36 patients with type 2 diabetes ( $n = 20$  and  $n = 16$ , respectively). While postprandial blood glucose and hemoglobin A<sub>1c</sub> improved in both groups, only sitagliptin improved the lipid profile at 1 or 3 h postprandially. Although its small size is a major limitation of this study, the results provide some interesting information about the metabolic effects of these two drugs in diabetic patients.

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

### Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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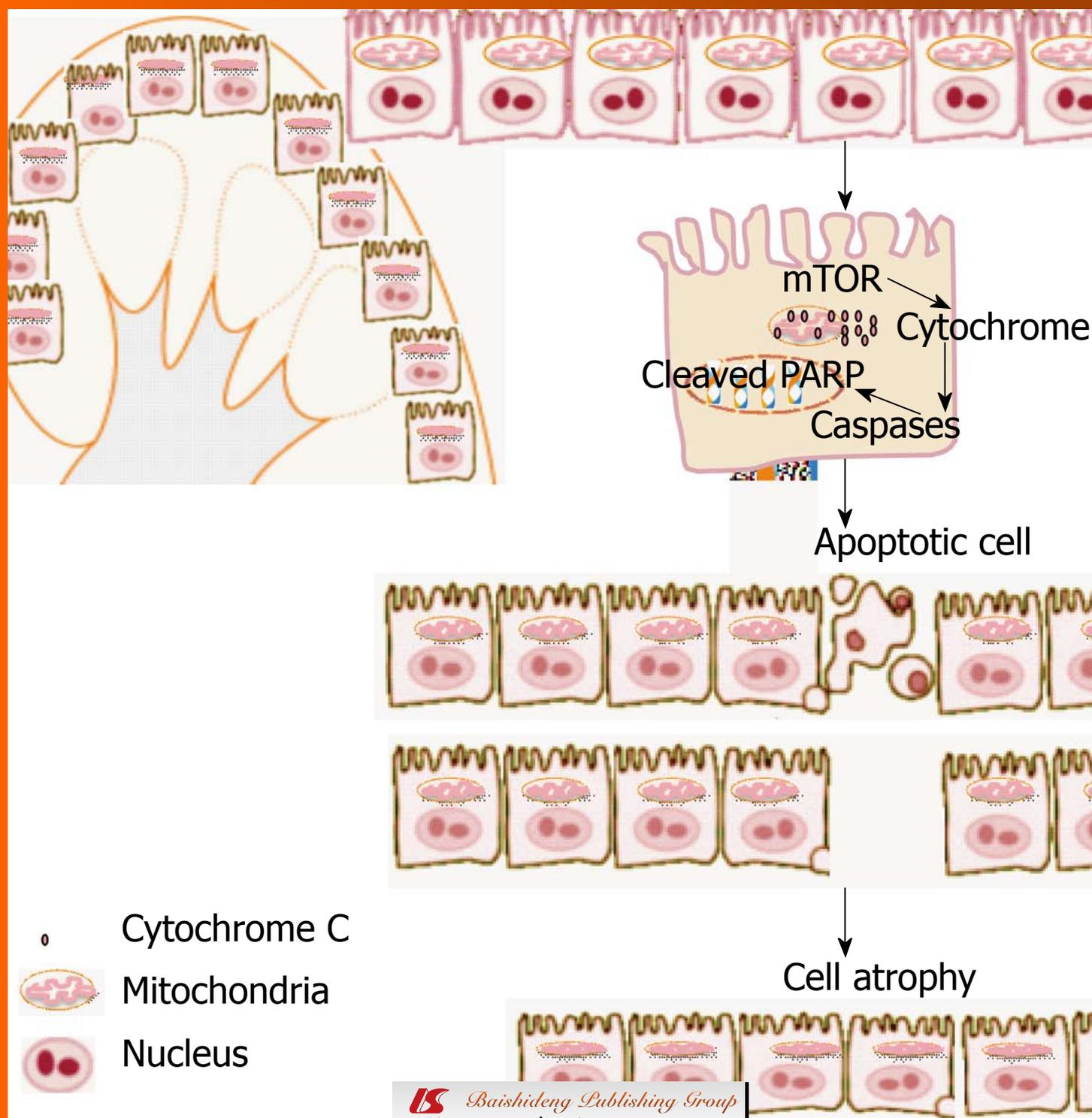
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## Contents

Bimonthly Volume 4 Number 2 April 15, 2013

- |                         |    |  |
|-------------------------|----|--|
| <b>FIELD OF VISION</b>  | 14 | Bariatric surgery as a treatment option in patients with type 2 diabetes mellitus<br><i>Spanou M, Tziomalos K</i>  |
| <b>TOPIC HIGHLIGHT</b>  | 19 | Bevacizumab for the management of diabetic macular edema<br><i>Stefanini FR, Arevalo JF, Maia M</i>  |
| <b>MINIREVIEWS</b>      | 27 | Diabetes and renal tubular cell apoptosis<br><i>Habib SL</i>   |
| <b>ORIGINAL ARTICLE</b> | 31 | Negative association between trunk fat, insulin resistance and skeleton in obese women<br><i>Greco EA, Francomano D, Fornari R, Marocco C, Lubrano C, Papa V, Wannenes F, Di Luigi L, Donini LM, Lenzi A, Aversa A, Migliaccio S</i> |
| <b>BRIEF ARTICLE</b>    | 40 | Parental transmission of type 2 diabetes mellitus in a highly endogamous population<br><i>Bener A, Yousafzai MT, Al-Hamaq AOAA, Mohammad AG, DeFronzo RA</i>   |

**APPENDIX** I-V Instructions to authors

**ABOUT COVER** Habib SL. Diabetes and renal tubular cell apoptosis.  
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## Bariatric surgery as a treatment option in patients with type 2 diabetes mellitus

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### Abstract

Type 2 diabetes mellitus (T2DM) is a leading cause of blindness, non-traumatic amputation and end-stage renal disease as well as a major cardiovascular risk factor. Tight glycemic control reduces the incidence of microvascular complications of T2DM whereas its effects on macrovascular complication are more controversial. However, glycemic targets are achieved by a minority of diabetic patients despite the availability of several antidiabetic agents. In the present commentary, we discuss the findings of two recent randomized studies that compared bariatric surgery with medical treatment in patients with uncontrolled T2DM. Both studies showed that bariatric surgery results in remission of T2DM in the majority of patients. However, both studies were limited to relatively young patients without comorbidities, had relatively short follow-up and did not assess the effects of surgery on T2DM complications. Moreover, the perioperative complications of bariatric surgery and its limited availability in some areas are additional barriers to the wider implementation of this therapeutic approach. On the other hand, the elucidation of the mechanisms underpinning the resolution of T2DM following bariatric surgery might result

in the development of novel, more effective pharmacotherapies for this common disease.

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**Key words:** Type 2 diabetes mellitus; Bariatric surgery; Roux-en-Y gastric bypass; Biliopancreatic diversion; Sleeve gastrectomy; Adjustable gastric banding

**Core tip:** In the present commentary, we discuss the findings of two recent randomized studies that compared bariatric surgery with medical treatment in patients with uncontrolled type 2 diabetes mellitus (T2DM). Both studies showed that bariatric surgery results in remission of T2DM in the majority of patients. However, both studies were limited to relatively young patients without comorbidities, had relatively short follow-up and did not assess the effects of surgery on T2DM complications.

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### COMMENTARY ON HOT TOPICS

Type 2 diabetes mellitus (T2DM) has become a global epidemic in the recent decades<sup>[1]</sup>. Diabetes mellitus affects 346 million people worldwide and T2DM accounts for 90% of the cases<sup>[1]</sup>. Moreover, T2DM is a leading cause of blindness, non-traumatic amputation and end-stage renal disease as well as a major risk factor for cardiovascular disease<sup>[1]</sup>.

Tight glycemic control reduces the risk for the microvascular complications of T2DM whereas its effects on macrovascular complications are more controversial<sup>[2-8]</sup>.

Methods to achieve glycemic control include lifestyle changes (diet and exercise) and pharmacotherapy with either oral or injectable agents, the latter primarily including insulin<sup>[9]</sup>. However, glycemic control progressively deteriorates during treatment with oral agents in the majority of patients as a result of the progressive decline in insulin secretion from pancreatic beta cells<sup>[10]</sup>. In addition, glycemic targets are achieved by a small minority of patients even in specialist centers<sup>[11,12]</sup>. Moreover, tight glycemic control is associated with increased risk for hypoglycemia and weight gain, resulting in decreased adherence to treatment, which in turn further worsens glycemic control<sup>[5-8]</sup>. In turn, suboptimal glycemic control is associated with increased risk for complications, particularly nephropathy and retinopathy<sup>[2,4,5]</sup>.

Given the limited efficacy of existing antidiabetic agents in achieving glycemic targets, bariatric surgery has been evaluated for the management of severely obese patients with T2DM and yielded promising results in uncontrolled studies<sup>[13]</sup>. Recently, two studies compared bariatric surgery with medical treatment in patients with uncontrolled T2DM<sup>[14,15]</sup>. In the first study, Mingrone *et al.*<sup>[14]</sup> evaluated two types of bariatric surgery, laparoscopic Roux-en-Y gastric bypass and open biliopancreatic diversion, in patients 30-60 years-old with a body mass index (BMI)  $\geq 35$  kg/m<sup>2</sup>, who had T2DM for  $\geq 5$  years and hemoglobin A1c (HbA1c) levels  $\geq 7\%$ . Patients with type 1 diabetes mellitus, severe diabetes complications, other severe medical conditions or previous bariatric surgery were excluded from the study<sup>[14]</sup>. Sixty patients were randomly assigned into three treatments: Roux-en-Y gastric bypass, biliopancreatic diversion and medical treatment (lifestyle modification, oral hypoglycemic agents and/or insulin)<sup>[14]</sup>. The primary endpoint was the rate of remission of T2DM at 2 years, defined as fasting plasma glucose levels  $< 100$  mg/dL (5.6 mmol/L) and HbA1c levels  $< 6.5\%$  for at least 1 year without pharmacologic treatment<sup>[14]</sup>. Diabetes remission was achieved at 2 years in 75 and 95% of patients who had undergone gastric bypass and biliopancreatic diversion, respectively. None of the patients assigned to medical treatment achieved T2DM remission ( $P < 0.001$  *vs* both surgery groups)<sup>[14]</sup>. Age, sex, baseline BMI, diabetes duration and weight change did not predict T2DM remission<sup>[14]</sup>. Weight loss was similar in the two surgical groups (approximately 33%) and smaller in the medical treatment group (4.7%)<sup>[14]</sup>. Regarding other cardiovascular risk factors, serum low density lipoprotein cholesterol (LDL-C) and triglyceride (TG) levels showed a similar reduction in the medical treatment and gastric bypass groups but decreased more in the biliopancreatic diversion group<sup>[14]</sup>. In contrast, serum high density lipoprotein cholesterol (HDL-C) levels showed a similar increase in the medical treatment and biliopancreatic diversion groups but increased more in the gastric bypass group<sup>[14]</sup>. Blood pressure (BP) decreased and the number of antihypertensive agents was reduced to a comparable extent in the three groups<sup>[14]</sup>.

In the second study, Schauer *et al.*<sup>[15]</sup> compared in-

tensive medical treatment alone and intensive medical treatment combined with either laparoscopic Roux-en-Y gastric bypass or laparoscopic sleeve gastrectomy in 150 patients 20-60 years old with a BMI between 27 and 43 kg/m<sup>2</sup>, and with HbA1c levels  $> 7\%$ . Patients with uncontrolled medical or psychiatric disorders or previous bariatric or complex abdominal surgery were excluded from the study<sup>[15]</sup>. The primary endpoint, the rate of patients with HbA1c levels  $\leq 6\%$  at 12 mo with or without antidiabetic medications, was achieved in 42% of patients who underwent gastric bypass, in 37% of patients who underwent sleeve gastrectomy and in 12% of patients in the medical treatment group ( $P = 0.002$  and  $P = 0.008$  for the comparison between medical treatment with gastric bypass and sleeve gastrectomy, respectively)<sup>[15]</sup>. Age, baseline BMI, diabetes duration and use of insulin did not predict the primary outcome<sup>[15]</sup>. Percentage weight loss was greater with gastric bypass than with sleeve gastrectomy (27.5% and 24.7%, respectively;  $P = 0.02$ ) whereas patients assigned to medical treatment lost less weight (5.2%;  $P < 0.001$  *vs* both surgical groups)<sup>[15]</sup>. In both surgical groups, serum high sensitivity C-reactive protein levels decreased and HDL-C levels increased compared with the medical treatment group<sup>[15]</sup>. In contrast, serum TG levels decreased only in the gastric bypass group compared with the medical treatment group<sup>[15]</sup>. Serum LDL-C levels and BP did not differ among groups after 12 mo but the use of lipid-lowering and antihypertensive medications declined significantly only in the surgical groups<sup>[15]</sup>.

Overall, both studies suggest that bariatric surgery is more effective in achieving glycemic control than medical treatment and results in T2DM remission (*i.e.*, no need for antidiabetic medications) in a sizeable proportion of patients<sup>[14,15]</sup>. The higher remission rates in the study by Pournaras *et al.*<sup>[16]</sup> might be due to differences in operative technique and the less stringent criteria for defining remission, the longer follow-up or the shorter duration of T2DM; on the other hand, the smaller sample size suggests the possibility of a type 1 statistical error<sup>[14,15]</sup>. In both studies, other cardiovascular risk factors, including dyslipidemia and hypertension, also improved substantially after bariatric surgery<sup>[14,15]</sup>. Importantly, the benefits of bariatric surgery appeared to be independent of the preoperative BMI<sup>[14,15]</sup> and, in the study by Schauer *et al.*<sup>[15]</sup>, to apply not only to patients with BMI  $> 35$  kg/m<sup>2</sup> but also to those with BMI 27-35 kg/m<sup>2</sup>. This finding suggests that current recommendations that propose bariatric surgery only for patients with T2DM with BMI  $> 35$  kg/m<sup>2</sup> might need to be modified<sup>[17]</sup>. The benefits of bariatric surgery were also independent of age (within the age range of 20-60 years-old)<sup>[14,15]</sup>. Diabetes remission rates were also independent of diabetes duration<sup>[14,15]</sup> whereas previous retrospective studies reported that patients with longer-lasting T2DM show lower rates of T2DM resolution after bariatric surgery<sup>[18]</sup>. Therefore, this finding should be interpreted with caution because both studies were rather small and probably underpowered to detect an association between T2DM remission rates and dia-

betes duration<sup>[14,15]</sup> and also because the variability of T2DM duration was very small in the study by Mingrone *et al.*<sup>[14]</sup> (mean duration, 6.0 ± 1.1 years). The findings of these trials are in agreement with previous uncontrolled studies that reported resolution of T2DM in 65%-83% of patients<sup>[13,18-22]</sup> and with a smaller study in 60 diabetic patients with BMI 30-40 kg/m<sup>2</sup> where laparoscopic adjustable gastric banding and medical treatment resulted in T2DM remission in 73% and 13% of patients, respectively<sup>[23]</sup>. In addition, these benefits add to the other positive effects of bariatric surgery including remission of other obesity-associated comorbidities such as hypertension, dyslipidemia, metabolic syndrome, chronic kidney disease, left ventricular hypertrophy, non-alcoholic fatty liver disease and obstructive sleep apnea<sup>[24,25]</sup>. Preliminary data from uncontrolled studies also suggest a reduction in cancer rates following bariatric surgery<sup>[26,27]</sup>. Bariatric surgery also appears to reduce the risk of T2DM in obese patients<sup>[28]</sup>. However, it should be noted that other studies did not show a beneficial effect of bariatric surgery on obesity-related comorbidities, including non-alcoholic fatty liver disease and obstructive sleep apnea<sup>[29,30]</sup>.

Is therefore bariatric surgery an alternative option for patients with T2DM? Probably not yet, for both medical and logistic reasons. First, bariatric surgery is infrequently associated with both short- and long-term complications, including mortality, even in experienced centers<sup>[31]</sup>. In the two described studies, there were no perioperative deaths but 6 patients (4.3%) required reoperation<sup>[14,15]</sup>. However, these studies were small, had a relatively short-term follow-up and were performed in experienced centers<sup>[14,15]</sup>. Perioperative mortality rates of bariatric surgery range between 0.10% and 0.35%<sup>[31]</sup>. Non-fatal perioperative complications, including anastomotic and staple line leaks, wound infections, pulmonary embolism and hemorrhage occur at higher rates (1.7%-3.1%) even though they are progressively becoming less frequent, mainly as a result of higher hospital volumes<sup>[32,33]</sup>. Second, it is still unclear whether bariatric surgery reduces cardiovascular events, even though uncontrolled studies suggested a cardiovascular morbidity and mortality benefit<sup>[26,34,35]</sup>. Third, existing randomized studies excluded patients with comorbidities and those older than 60 years, who constitute the majority of patients with T2DM<sup>[14,15]</sup>. Finally, the lack of experienced surgeons in many areas and the cost of bariatric surgery are additional barriers to the wider implementation of this treatment, even though the cost of bariatric surgery might compare favorably with the costs of the lifelong management of diabetes and of its micro- and macrovascular complications<sup>[36-38]</sup>.

In conclusion, bariatric surgery might be considered in relatively young patients with uncontrolled T2DM despite adequate pharmacological treatment, without comorbidities, and with BMI > 35 kg/m<sup>2</sup>. Current guidelines state that bariatric surgery may be considered for adults with BMI ≥ 35 kg/m<sup>2</sup> and T2DM, especially if the diabetes or associated comorbidities are difficult to control with lifestyle and pharmacological therapy (level

of evidence B)<sup>[17]</sup>. They also state that there is currently insufficient evidence to generally recommend surgery in patients with BMI < 35 kg/m<sup>2</sup> outside of a research protocol (level of evidence E)<sup>[17]</sup>. Even though existing guidelines do not mention specific contraindications for bariatric surgery, it is clear that the risk of peri- and postoperative complications should be balanced against the benefits of bariatric surgery<sup>[17]</sup>. However, given the high and rising prevalence of T2DM as well as the lack of long-term data on safety and efficacy of bariatric surgery, this treatment will probably have limited impact on the T2DM epidemic. On the other hand, weight loss cannot entirely explain the beneficial effects of bariatric surgery because these occur soon after the operation and before maximum weight loss is achieved<sup>[15-15]</sup>. Changes in the bioavailability of gut hormones, fat malabsorption and improvement of insulin resistance might also play a role<sup>[39-45]</sup>. In contrast, the exclusion of proximal small intestine does not appear to contribute to the improvement in glucose homeostasis<sup>[45]</sup>. On the other hand, accumulating data suggest that newer classes of antidiabetic agents, including thiazolidinediones and incretin-based agents, might delay the decline in beta cell function by alleviating glucolipototoxicity<sup>[46]</sup>. Recent data suggest that bariatric surgery also has a beneficial effect on beta cell function<sup>[44]</sup>. The extensive discussion of the mechanisms involved in the remission of T2DM after bariatric surgery is beyond the scope of this commentary; several comprehensive reviews on the topic have been published recently<sup>[47,48]</sup>. The elucidation of the pathophysiologic mechanisms underpinning the resolution of T2DM and other obesity-associated comorbidities after bariatric surgery might lead to the development of novel and more effective pharmacotherapies for these common diseases.

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## Bevacizumab for the management of diabetic macular edema

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### Abstract

Diabetic retinopathy (DR) is a leading cause of vision loss in the working-age population and is related to 1%-5% of cases of blindness worldwide. Diabetic macular edema (DME) is the most frequent cause of DR vision loss and is an important public health problem. Recent studies have implicated vascular endothelial growth factor (VEGF) in DR and DME pathogenesis, as well as provided evidence of the benefits of anti-VEGF agents for the management of such conditions. Despite the benefits of intravitreal ranibizumab injection for the management of DME, the cost-effectiveness of intravitreal bevacizumab therapy has gained increasing interest in the scientific community. This review summarizes the studies examining bevacizumab for the management of DME, focusing on the efficacy and duration of the clinical

benefits of decreasing DME and the improvement of best-corrected visual acuity (BCVA). There is strong evidence that intravitreal bevacizumab injection therapy has a good cost-effective profile in the management of DME and may be associated with laser photocoagulation; however, its clinical superiority in terms of the duration of DME regression and the improvement of BCVA compared with intravitreal ranibizumab and other intravitreal anti-VEGF therapies remains unclear and deserves further investigation.

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**Key words:** Diabetic macular edema; Bevacizumab; Anti-vascular endothelial growth factor; Diabetic retinopathy

**Core tip:** This review summarizes the studies examining bevacizumab for the management of diabetic macular edema (DME), focusing on the efficacy and duration of the clinical benefits of decreasing DME and the improvement of best-corrected visual acuity.

Stefanini FR, Arevalo JF, Maia M. Bevacizumab for the management of diabetic macular edema. *World J Diabetes* 2013; 4(2): 19-26 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i2/19.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i2.19>

### INTRODUCTION

Diabetic retinopathy (DR) is a leading cause of vision loss in working-age patients around the world. DR is related to 1% of all cases of blindness worldwide, and it may be related to 5% of blindness in some countries<sup>[1,2]</sup> (Figure 1). The main cause of vision impairment in diabetic patients is diabetic macular edema (DME)<sup>[3-5]</sup>. DME may occur at any stage of non-proliferative or proliferative DR<sup>[6,7]</sup>. Macular edema is divided into two types: focal and diffuse. Focal macular edema is caused by focal leak-

age from microaneurysms and dilated retinal capillaries with abnormal permeability. Complete or partial rings, as a circinate pattern of hard exudates, often demarcate the macular edema<sup>[8]</sup> (Figure 2A). In diffuse macular edema, generalized leakage from dilated capillaries is observed throughout the posterior pole (Figure 2B). Occlusion of a portion of the capillary bed causes dilation of the patent capillaries, which tend to leak, leading to edema<sup>[9]</sup>. The risk factors associated with diffuse macular edema are systemic hypertension, adult-onset diabetes mellitus and poor blood glucose control, cardiovascular disease, impaired renal function, increased number of retinal microaneurysms, advanced retinopathy and vitreomacular traction<sup>[9,10]</sup>. It is estimated that DME occurs in 3% to 6% of all patients with diabetes aged 18 or older<sup>[11]</sup>. A large epidemiological study indicated that macular edema was present in 26% of the study patients with DR<sup>[12]</sup>.

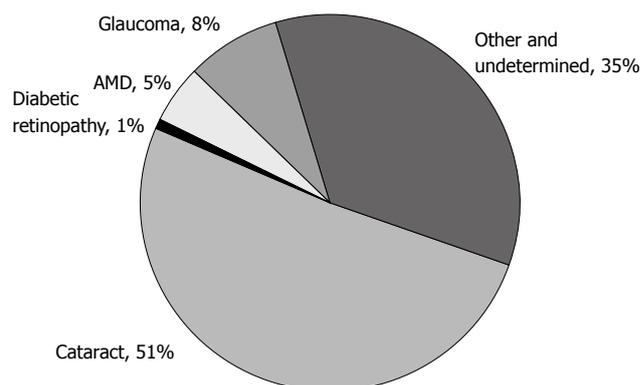
The most efficient tool for preventing vision loss from DR is screening and identification of at-risk patients, along with regular office visits to educate patients on the importance of tight blood sugar and blood pressure control in both type 1 and type 2 diabetes<sup>[3]</sup>.

Once a patient develops DME, the gold standard treatment in recent decades has been macular photocoagulation (MPC) using the laser technique, which reduces the risk of moderate visual loss by approximately 50% (Figure 3)<sup>[13]</sup>. A review of the data from the Early Treatment DR Study (ETDRS) demonstrated that approximately 40% of the patients who demonstrated improvement with focal laser treatment and a baseline best-corrected visual acuity (BCVA) worse than 20/40 had gained 6 or more letters at 3-year post follow-up<sup>[13,14]</sup>. Recently, the Diabetic Retinopathy Clinical Research Network (DRCR.net) has demonstrated BCVA improvement of more than 5 letters of vision in 51%, 47% and 62% of eyes treated with MPC after 1, 2 and 3 years of follow-up, respectively<sup>[5,15-17]</sup>.

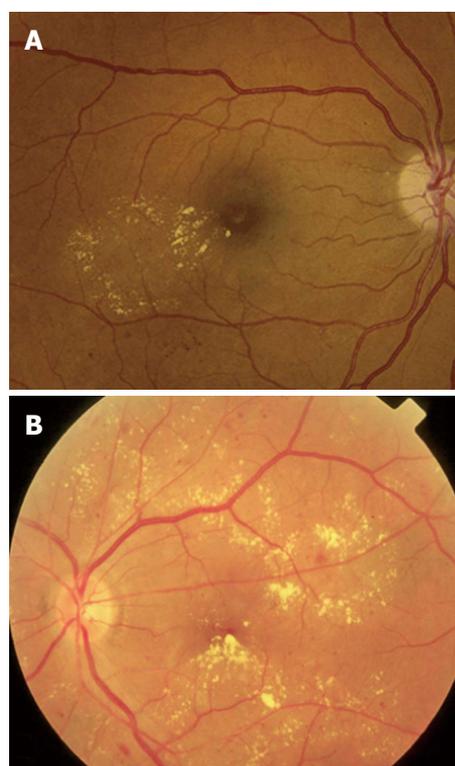
## VASCULAR ENDOTHELIAL GROWTH FACTOR INHIBITORS AND DME

In recent years, alternative or adjunct treatments for DME have been studied, and various pharmacological compounds are under investigation, such as intravitreal triamcinolone acetonide (IVTA) and therapies using inhibitors of vascular endothelial growth factor (VEGF)<sup>[4]</sup>. Studies performed by DRCR.net demonstrated that despite the early benefits of intravitreal injection of 4 mg of triamcinolone acetonide (TA), the BCVA and retinal thickening at 4 mo compared with a 1-mg TA dose or with focal/grid photocoagulation, the final mean BCVA at 2 and 3 years was better in the MPC group<sup>[15,16]</sup>.

VEGF expression and signaling are deregulated in DR, and VEGF is an important mediator of blood retinal barrier breakdown, which leads to fluid leakage below the macula and the development of macular edema. Therefore, at present, treatment with anti-VEGF agents is one of the most promising approaches for the treatment of

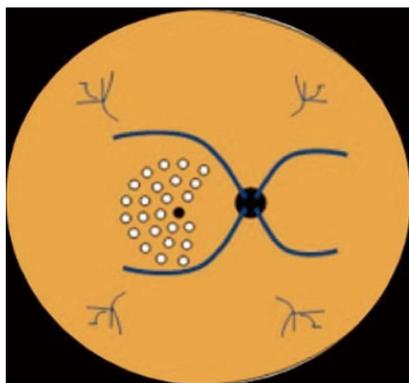


**Figure 1** Pie chart displaying the distribution of global causes of blindness. Although cataracts are responsible for more than half of the cases, they are potentially reversible. When considering the causes of permanent vision impairment, diabetic retinopathy contributes significantly to 1%-5% of cases of blindness. In addition, diabetic retinopathy is the major cause of irreversible blindness in the working-age patients worldwide. AMD: Age-related macular disease.



**Figure 2** Clinical patterns of diabetic macular edema. A: Focal macular edema marked by focal leakage from microaneurysms and dilated retinal capillaries with abnormal permeability, making a complete ring as a localized circinate pattern of hard exudates; B: Diffuse macular edema, characterized by hard exudates with generalized leakage from dilated capillaries throughout the posterior pole.

vision loss due to DME<sup>[18,19]</sup>. Several studies have been conducted that have addressed the efficacy and safety of anti-VEGF agents, including ranibizumab (Lucentis, Genentech, Inc., United States), pegaptanib (Macugen, OSI/Eyetech, United States), and aflibercept (EYLEA; Regeneron, United States) and bevacizumab (Avastin, Genentech, Inc., United States), in the treatment of DME.



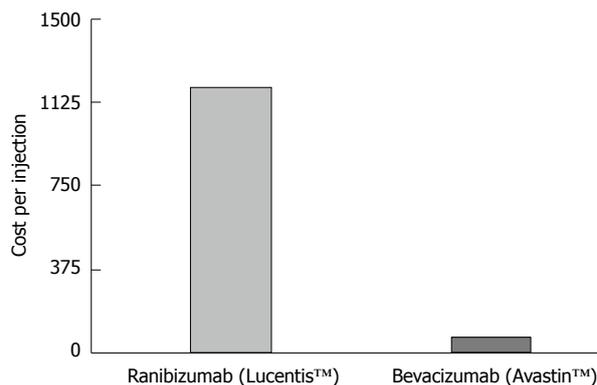
**Figure 3** Macular area treated by laser photocoagulation using the scheme proposed by the Early Treatment Diabetic Retinopathy Study.

It has been shown that pegaptanib inhibits VEGF permeability effects<sup>[20,21]</sup>. The VEGF Inhibition Study in Ocular Neovascularization trial established the safety and efficacy in neovascular age-related macular disease (AMD)<sup>[22]</sup>. For DME, the efficacy and safety of 0.3 mg of pegaptanib sodium *vs* sham injections was studied in a phase-2/3, multicenter, randomized, double-blinded trial<sup>[23]</sup>. After 102 wk, the pegaptanib group presented significantly better results than the sham injection group in BCVA change, letters gained and reduced need for focal/grid laser photocoagulation.

Recently, 2 mg/0.05 mL aflibercept (EYLEA; Regeneron, United States) received regulatory approval from the Food and Drug Administration (FDA) for the treatment of neovascular AMD. For management of DME, a multicenter, randomized, double-masked, phase-2 clinical trial, the DA VINCI Study, tested different dosing regimens of aflibercept (VEGF Trap-Eye) and compared them with laser photocoagulation: 0.5 mg every 4 wk, 2 mg every 4 wk, 2 mg for the 3 initial doses then every 8 wk, 2 mg for the 3 initial doses then as needed. Subjects in the VEGF Trap-Eye groups experienced mean reductions in central retina thickness and, at their 6-mo follow-up, had better results for BCVA than those who were treated with laser photocoagulation. However, it is important to note that a considerable number of re-injections were necessary.

The drug was well tolerated. The phase-3 trials on aflibercept in patients with visual loss due to DME are ongoing<sup>[7,24]</sup>.

Ranibizumab is approved for the treatment of neovascular AMD and just received FDA approval (August 2012) for the treatment of visual impairment due to DME, based on the RIDE and RISE clinical trials. Several clinical trials have been performed examining the use of ranibizumab for the treatment of visual impairment due to DME. The RESTORE study demonstrated superiority after 12 mo of ranibizumab monotherapy (0.5 mg) administered as needed or as an adjunct to laser photocoagulation *vs* laser monotherapy<sup>[25,26]</sup>. The READ-2 study found that ranibizumab (0.5 mg) alone or in combination with laser photocoagulation improved BCVA over 2 years



**Figure 4** Cost per injection of ranibizumab (Lucentis™ Genentech, United States), in the treatment of diabetic macular edema, compared with the cost per injection of bevacizumab (Avastin, Roche, United States). The cost is, in average, 20-fold higher in for treatment with ranibizumab than bevacizumab. Depending on the country, this difference may vary from 20× to 50×.

in DME patients<sup>[27]</sup>. RIDE and RISE, two identically designed, parallel, double-blinded, 3-year clinical trials that were sham-treatment controlled for 24 mo had preliminary results that demonstrated that patients who received 0.3 mg of ranibizumab experienced significant, early and sustained improvements in vision. The DRCR.net conducted a study to investigate the role of ranibizumab and also steroid treatment combined with laser photocoagulation. The 2-year results of this study indicated that 0.5 mg of ranibizumab administered as needed and combined with laser therapy produced a rapid and sustained improvement in the BCVA of patients with DME compared with laser treatment<sup>[28]</sup>.

## BEVACIZUMAB FOR DME

Bevacizumab is a full-size, humanized, recombinant monoclonal immunoglobulin G antibody that inactivates all VEGF isoforms. It is approved as an anti-VEGF agent for the systemic treatment of metastatic colorectal cancer, but its use for ocular diseases is off-label. Intravitreal bevacizumab (IVB) has been more widely utilized, primarily due to its low cost, safety and positive clinical effects in case studies and retrospective studies (Figure 4)<sup>[29-31]</sup>. The widespread use of IVB for the exudative form of AMD as well as the evidence of positive clinical effects in the management of DME<sup>[28,32,33]</sup> have resulted in the formal evaluation of its safety and efficacy in the management of DME<sup>[34]</sup>.

## RESULTS AND DISCUSSION

The DRCR.net conducted a randomized study of 121 eyes over a 12-wk period<sup>[33]</sup>. It consisted of five treatment arms: (1) focal photocoagulation; (2) two intravitreal injections of 1.25 mg of bevacizumab at 0 and 6 wk; (3) two intravitreal injections of 2.5 mg of bevacizumab at 0 and 6 wk; (4) 1.25 mg of bevacizumab at week 0 followed by a sham injection at 6 wk; and (5) 1.25 mg of bevacizumab at 0 and 6 wk combined with focal photocoagula-

tion at 3 wk. The majority of eyes, 69%, were refractory to previous treatment for DME. The eyes of two groups that received two bevacizumab injections without laser, 2 and 3, had a significant BCVA improvement over the laser-only group 1, and this difference persisted through the 12 wk. These two groups also had a greater improvement in central subfield thickness at the 3-wk visit. No differences were observed between groups 2 and 3 (1.25-mg and 2.5-mg doses, respectively). The single injection group had no advantage over the photocoagulation group in this study. Group 5, which combined bevacizumab with photocoagulation, had results comparable with laser-only treatment. This study suggested that bevacizumab was an effective drug for the management of DME as a primary treatment and also for refractory eyes. Safety data were reported for 24 wk, and no safety concerns were detected. Two trends were identified: (1) the eyes that received primary treatment had greater improvement ( $P = 0.04$ ) than the refractories; and (2) the presence of subretinal fluid at the initial therapy [measured by optical coherence tomography (OCT)] may be associated with a greater improvement in BCVA ( $P = 0.06$ ).

The DRCR.net study identified no difference between 1.25 mg and 2.5 mg of bevacizumab, and similar outcomes have been previously reported by other colleagues in retrospectively designed studies<sup>[35,36]</sup>. One of these studies involved three initial injections monthly and a follow-up period of 6 mo<sup>[33]</sup>; another study followed the same design but with a 12-mo follow-up<sup>[34]</sup>. Both studies demonstrated significant reductions in central foveal thickness (CFT) by OCT evaluation and also significant improvements in BCVA<sup>[33,34]</sup>. There were statistically similar outcomes for the two study groups throughout the 6 initial months and a trend toward recurrence of edema at the 1-year follow-up, suggesting a trend of reducing the CFT during the 2-3 mo following the intravitreal bevacizumab injection (IVBI)<sup>[33]</sup>.

Another study focused on IVB for DME investigated a remarkably diverse group of eyes, with no exclusions based on previous treatment, ischemia, or poor initial BCVA<sup>[37]</sup>. The study consisted of a noncomparative trial of 1.25 mg of bevacizumab at baseline, with subsequent re-treatment based on improvement in OCT or BCVA response to the initial injection. At 6 mo, there was no significant improvement in mean BCVA, but there were significant decreases in the mean CFT according to OCT evaluation. Although some characteristics of this study led to difficulty in analyzing its results, such as the diverse baseline data and a variable number of treatments, the results corroborated the idea that bevacizumab should be the object of further studies for eyes with DME refractory to previous treatments, as this therapeutic approach was able to decrease the CFT as measured by OCT.

When investigating the long-term effects of intravitreal bevacizumab in patients with chronic diffuse DME, Kook *et al.*<sup>[38]</sup> observed a decrease in central macular thickness (CMT) and again in BCVA following repeated intravitreal injections of bevacizumab, even in cases with

chronic diffuse ischemic DME.

Bonini-Filho *et al.*<sup>[39]</sup> performed a pilot study of IVB-treatment for macular edema in ten eyes with severe capillary loss. The treatment used 1.5-mg dosing, and all ten eyes underwent an injection at baseline. Re-treatment at follow-up visits was based on the presence of intraretinal or subretinal fluid on OCT. After 54 wk, the CMT and BCVA improved significantly. No progression of capillary loss was observed in fluorescein angiogram at the end of the study.

The BOLT Study, a prospective, randomized, blinded, single-center study, compared IVB and macular laser photocoagulation in patients with persistent CSME after at least one macular laser treatment<sup>[40]</sup>. Eighty eyes were randomized into a bevacizumab treatment group (with injections every 6 wk), with a minimum of 3 and a maximum of 9 injections, or a photocoagulation group, with sessions every 4 mo and a minimum of 1 and a maximum of 4 treatments. After 1 year, the mean BCVA measured by ETDRS evaluation increased in the bevacizumab group and deteriorated in the laser group. The CMT results were also favorable for the bevacizumab group. The median number of injections in this first year was 9 in the bevacizumab group, and the median number of laser treatments was 3.

The 2-year outcome report of the BOLT Study was published recently and presented similar results to the first year report<sup>[41]</sup>. The mean ETDRS equivalent Snellen was 20/50 in the bevacizumab group and 20/80 in the laser group ( $P = 0.005$ ). The bevacizumab group gained a median of 9 ETDRS letters *vs* 2.5 letters for the laser treatment group ( $P = 0.005$ ), with a mean gain of 8.6 letters for bevacizumab *vs* a mean gain of 0.5 letters for the laser group. Among the eyes treated with bevacizumab, 32% gained at least 15 letters *vs* 4% for the laser-treated eyes ( $P = 0.004$ ). The percentage of patient eyes that lost fewer than 15 letters in the macular laser treatment group was 86% *vs* 100% for the bevacizumab group ( $P = 0.03$ ). At 2 years, the CMT decreased significantly in both groups. At the 2-year follow-up, the median number of injections was 13, and the median number of laser treatments was 4.

In addition to MPC, some of the largest trials published examining bevacizumab use for DME have compared intravitreal bevacizumab and intravitreal triamcinolone (IVT).

Ahmadieh *et al.*<sup>[30]</sup> conducted a 24-wk trial randomizing 115 eyes to one of three study arms: a bevacizumab-only arm, an IVTA/bevacizumab combination arm, and a placebo arm. The two treatment arms received three 1.25-mg bevacizumab injections every 6 wk, and the IVTA/bevacizumab group received an additional injection of 2 mg of triamcinolone at the baseline visit only. No difference in BCVA or CMT was detected between the bevacizumab and IVTA/bevacizumab groups.

In a study performed by Faghihi *et al.*<sup>[42]</sup>, IVB-only was compared with bevacizumab associated to triamcinolone and with MPC in eyes with no history of treatment.

Dosings of 1.25 mg of bevacizumab and 2 mg of triamcinolone were used, and injections were performed at the baseline visit only. The three groups had significant improvements in CMT at both the 6- and 16-wk visits *vs* baseline. A similar trend was observed for BCVA; the bevacizumab group outperformed the laser group in CMT and BCVA at 6 wk but not at 16 wk. The bevacizumab/IVTA group outperformed the laser group in CMT and BCVA at both 6 and 16 wk.

A randomized clinical trial comparing IVB injection alone or in combination with IVTA *vs* macular laser photocoagulation as a primary treatment for DME was conducted by Soheilian *et al.*<sup>[5,43]</sup>, and the 2-year outcomes results were recently published. In total, 150 eyes were randomly assigned to 1 of the 3 study arms: the 1.25-mg IVB group; the IVB/IVT group, with 1.25 mg of IVB and 2 mg of IVT; and the macular laser group. There was significant superiority of visual acuity improvement in the IVB group after 6 mo, but this was not sustained after 24 mo. The mean BCVA improvement was greater in the IVB group than in the other groups and also in the IVB/IVT group compared with the laser group. The same was noted for the reduction of CMT, which was more evident in the IVB group compared to the other groups. However, the difference among the groups was not significant, which may be related to some methodological aspects, such as the 3-mo re-treatment intervals, when indicated, or the missing data in 24.6% of the cases at the final follow-up.

In a retrospective study, Wu *et al.*<sup>[44]</sup> aimed to identify OCT patterns in diabetic DME that were predictive of visual outcomes after IVBIs. Thirty-one eyes with clinically significant DME<sup>[13]</sup> and without previous treatment underwent complete ophthalmic examination and OCT. The eyes were classified into 4 groups, based on the cross-sectional retinal morphologies, by using OCT features: diffuse retinal thickening, cystoid macular edema (CME), serous retinal detachment and vitreomacular interface abnormalities. The minimum required follow-up was 3 mo. Changes in CMT and total macular volume after IVB injections were evaluated as well as the BCVA. Patients with CME exhibited greater improvement in all evaluated parameters compared with other groups. The study concluded that OCT patterns in DME may be helpful in deciding the best treatment and predicting the outcome after IVBI. In addition, the study indicates that IVBI could be a primary therapeutic modality for CME<sup>[44]</sup>. Similar results were found in a retrospective study conducted by Roh *et al.*<sup>[32]</sup>.

The Pan-American Collaborative Retina Study Group has published the 24-mo results of a study examining intravitreal bevacizumab as the primary treatment for diffuse DME (DDME). For these retrospective, multicenter, interventional, comparative case series, the clinical data of 139 eyes with DDME at 11 centers from 8 countries were reviewed. All of the eyes were treated with off-label IVB with at least 1 intravitreal injection of 1.25 or 2.5 mg of bevacizumab. The dose received at baseline was the



**Figure 5** Diffuse diabetic macular edema treated with bevacizumab. In the left figure, the clinical fundus photograph shows the macular edema and hard exudates at the foveal center. In the right figure, a series of optical coherence tomographs (OCTs) taken at a 24-mo follow-up can be observed. The OCT image at baseline shows the intraretinal fluid with increased central macular thickness (CMT) and best-corrected visual acuity (BCVA) = 20/100. One month after the first injection, improvement in both BCVA and CMT was observed. This result was maintained throughout the 24-mo follow-up period after six injections and with final central macular thickness within normal limits without intraretinal fluid and the improvement of BCVA to 20/30. No laser photocoagulation was performed in this case.

same dose delivered throughout the study. The exclusion criteria were as follows: patients with DDME that were treated with laser photocoagulation or intravitreal triamcinolone previously, macular ischemia, intraocular inflammation, a prior history of vitreoretinal surgery or cataract surgery within the past 6 mo, uncontrolled intraocular pressure, and the presence of an epiretinal membrane or vitreomacular traction syndrome. Each patient underwent BCVA measurement with ETDRS charts, ophthalmic examination and OCT at baseline and 1, 3, 6, 12 and 24 mo after the initial injection. Fluorescein angiography was performed at the discretion of the examiner (usually every 6 mo). Patients received re-injections whenever there was a recurrence of DDME.

One month after the initial bevacizumab injection, improvements in the BCVA and CMT measurements were observed, and these significant changes continued during the 24-mo follow-up period. The improvement of the BCVA and OCT from one study after 6 injections during the 2-year period is shown (Figure 5). BCVA analysis demonstrated that after 24 mo, 72 (51.8%) eyes improved 2 or more ETDRS lines, 62 (44.6%) eyes remained stable, and 5 (3.6%) eyes decreased 2 or more ETDRS lines of BCVA. A twenty-four-month OCT analysis indicated that CMT measurements decreased from  $446.4 \pm 154.4 \mu\text{m}$  to  $279.7 \pm 80 \mu\text{m}$ . The mean number of IVB injections per eye was 5.8 (range, 1-15 injections) at a mean interval of  $12.2 \pm 10.4$  wk. The data analysis of BCVA and CMT found no significant differences between the 1.25- and 2.5-mg dose groups<sup>[45]</sup>.

A systematic review of IVBI for the treatment of primary DME was conducted by Yilmaz *et al.*<sup>[34]</sup> and published in 2011. The review compared IVB injection *vs* MPC *vs* a combination IVB/IVTA injection in improving the BCVA of patients without previous treatment for

DME<sup>[34]</sup>. The review included four randomized clinical trials comparing IVB injection with macular laser and three of them also comparing IVB injection with IVB/IVTA. The outcomes indicated that IVB injection is effective in improving BCVA in primary DME for 6 wk, but the benefits are no longer present at 12 wk after injection. IVTA had no detectable adjunctive effect.

Throughout the discussion of this systematic review, various limitations may be responsible for these observed outcomes, which somewhat contradict the trends shown in previous studies. First, this review was limited to four randomized controlled trials, and all of them had varied baseline characteristics. The DRCR.net study provided BCVA and CMT values that were not estimable in our analysis because there was a mixture of patients with and without prior treatment for DME. However, that study was included in the systematic review to emphasize that patients from IVB groups did improve in their BCVA and CMT values compared to the laser group. Another relevant aspect is that a decrease in efficiency may be related to the cessation of treatment in those studies in which just one injection was performed. The DRCR.net demonstrated that the improvement results were sustained for 12 wk with two IVB injections.

Therefore, the limitations of this analysis may corroborate the idea that IVB is effective in treating primary DME; however, IVB should not be considered the first line of treatment.

The safety of the intravitreal use of bevacizumab has also been studied. A retrospective study involving 1173 patients who received intravitreal bevacizumab and were followed for 12 mo is likely the largest series regarding the use of bevacizumab in DME. In this study, the following adverse effects were observed: seven cases of acute elevation of blood pressure, six strokes, five myocardial infarctions, five deaths, seven cases of bacterial endophthalmitis, seven cases of tractional retinal detachment (TRD), and four cases of uveitis<sup>[46]</sup>. These numbers were similar to those found in the prospective, controlled studies of the other anti-VEGF agents<sup>[5]</sup>.

TRD in proliferative diabetic retinopathy following intravitreal bevacizumab may happen because of natural history or rapid neovascular involution with accelerated fibrosis and posterior hyaloidal contraction as a response to decreased levels of VEGF. Arevalo *et al.*<sup>[47]</sup>, in a retrospective review, identified a 5.2% incidence of development or progression of TRD after treatment with intravitreal bevacizumab. Therefore, treatment with bevacizumab for patients with proliferative DR and DME must be cautiously applied, especially in cases with elevated glycosylated hemoglobin, patients with type 1 diabetes with poor glycemic control, patients without previous PRP or refractory to this treatment and the presence of areas of isolated TRD.

Although delivered intravitreally, anti-VEGF drugs can potentially circulate systemically<sup>[19]</sup>. Systemic side-effects such as arterial thromboembolism, gastrointestinal perforation, hemorrhage, hypertensive crisis, and nephrot-

ic syndrome are the main safety concerns surrounding the use of intravenous bevacizumab in patients with a diagnosis of colorectal cancer and other important systemic comorbidities.

## CONCLUSION

There is growing evidence that IVBI is safe and effective for the treatment of DME, both for cases with no prior treatment as well as for refractory eyes. The rationale of the current trend of using a combination therapy of IVBI with laser photocoagulation is based on the fast recovery of macular anatomy/BCVA related to prompt VEGF inhibition (due to the IVBI) associated with the long-term effects of laser (that may decrease the necessity of IVBI due to the sustained anti-VEGF effects of laser scars).

Comparing the effects of bevacizumab and other VEGF inhibitors is difficult; however, the cost-effectiveness and safety of IVBI is certainly the most important benefit of such treatment in comparison to all commercially available anti-VEGF therapies.

To optimize the management of DME, more studies should be performed to confirm its effectiveness and the duration of its benefits and to establish guidelines for the mean number and periodicity of IVBIs, either in isolation or combined with laser photocoagulation.

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## Diabetes and renal tubular cell apoptosis

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### Abstract

Apoptosis contributes to the development of diabetic nephropathy, but the mechanism by which high glucose induces apoptosis is not fully understood. Apoptosis of tubular epithelial cells is a major feature of diabetic kidney disease, and hyperglycemia triggers the generation of free radicals and oxidant stress in tubular cells. Hyperglycemia and high glucose *in vitro* also lead to apoptosis, a form of programmed cell death. High glucose similar to those seen with hyperglycemia in people with diabetes mellitus, lead to accelerated apoptosis, a form of programmed cell death characterized by cell shrinkage, chromatin condensation and DNA fragmentation, in variety of cell types, including renal proximal tubular epithelial cells.

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**Key words:** Tubular cells; Renal; Apoptosis; Diabetes

**Core tip:** Apoptosis contributes to the development of diabetic nephropathy, but the mechanism by which high glucose induces apoptosis is not fully understood. High glucose similar to those seen with hyperglycemia in people with diabetes mellitus, lead to accelerated apoptosis, a form of programmed cell death character-

ized by cell shrinkage, chromatin condensation and DNA fragmentation, in variety of cell types, including renal proximal tubular epithelial cells.

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### DIABETES AND RENAL TUBULAR CELL APOPTOSIS

Diabetes is the leading cause of end-stage renal failure in most developed countries. Although vascular and glomerular injuries have been considered the main features of diabetic kidney diseases, tubular atrophy is also plays a major role in the disease<sup>[1]</sup>. Diabetes induces early signs of tubular dysfunction<sup>[2]</sup>. In addition, diabetic kidneys are particularly prone to acute tubular necrosis in diverse clinical situations, such as post-cardiac surgery<sup>[3]</sup>. Hyperglycemia, by itself, is an independent risk factor for acute tubular necrosis under these conditions<sup>[3]</sup>. Hyperglycemia triggers the generation of free radicals and oxidant stress in tubular cells<sup>[4,5]</sup>. Reactive oxygen species are considered to be important mediators for several biologic responses, including proliferation, extracellular matrix deposition and apoptosis<sup>[6]</sup>. Apoptosis, a form of programmed cell death characterized by cell shrinkage, chromatin condensation and DNA fragmentation, which, can be induced by various stimuli<sup>[7]</sup>. High glucose concentration promotes apoptosis in variety of cell types including proximal tubular epithelial cells<sup>[5,8]</sup>. The mechanism by which hyperglycemia leads to apoptosis is not completely understood.

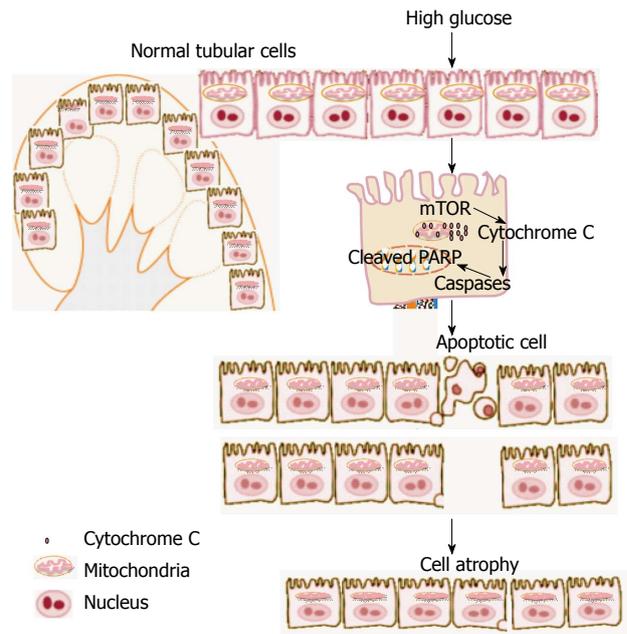
A high glucose concentration of 30 mmol/L for 18-48 h has been shown to induce apoptotic changes in HK2 cells via an increase in oxidative stress<sup>[8]</sup>. Prolonged exposure (1-13 d) of proximal tubular epithelial cells to hyperglycemic environment has been shown to inhibit cell proliferation and induce growth arrest or cellular

apoptosis<sup>[8-12]</sup>. These cellular effects are caused by the activation of a network of intracellular signaling pathways and include the phosphatidylinositol 3 kinase (PI3 kinase)/adams kara taylor (AKT) signaling pathway<sup>[13]</sup>. Activation of PI3 kinase and phosphorylation of serine/threonine kinase AKT/protein kinase B (PKB) by insulin, insulin like growth factors in human embryonic 293 (HEK-293) and HeLa cells lead to inactivation of tuberlin by phosphorylating at Ser939, Ser1086/1088 and Thr1422<sup>[14,15]</sup>. In addition, phosphorylation of tuberlin at Ser939 and Thr1422 in response to PDGF and insulin stimulation in a PI3K-dependent manner has been reported in NIH-3T3 and HEK-293 transfected with flag-tuberlin<sup>[16]</sup>. Moreover, high glucose has shown to phosphorylate tuberlin in renal cells<sup>[13]</sup>.

Tuberlin, which is the product of tumor suppressor gene, *TSC-2*<sup>[17]</sup> normally, exists in an active state physically bound to hamartin, the product of *TSC-1* gene to form a stable complex<sup>[18]</sup>. These two proteins function within the same mTOR signaling pathway. mTOR is a serine/threonine kinase involved in numerous cell processes linked to cell growth control, like cell cycle progression, transcription and translation control as well as nutrient uptake<sup>[19]</sup>. Loss of *TSC-2* function either by *TSC-2* or *TSC-1* deficiency leads to constitutive activation of mTOR and downstream signaling pathways due to increased levels of GTP-bound Rheb<sup>[20,23]</sup>. Therefore tuberlin, through its Rheb-GAP activity, is a critical negative regulator of mTOR under physiological conditions<sup>[24,25]</sup>. mTOR phosphorylates p70S6K (p70 ribosomal protein S6 kinase) on Thr389, which correlates with the activation of p70S6kinase<sup>[24,26]</sup>, while over-expression of *TSC-2* suppresses phosphorylation and activation of p70S6K on residue Thr389<sup>[14,16]</sup>. In addition, several studies have shown that Akt/mTOR pathway is activated in diabetes and this activation is redox dependent in different cell types<sup>[27-29]</sup> including renal cells<sup>[13]</sup>.

Previous reports have shown that the serine/threonine kinase, mTOR to be involved in the phosphorylation/inactivation of Bcl-2 in microtubules treated with apoptotic agents<sup>[30]</sup>. Bcl-2 plays a central role in monitoring the genetic programs of the organism<sup>[31,32]</sup>. Bcl2 related proteins comprise a family of positive and negative regulators of apoptosis. Bcl-2 and its close homolog Bcl-XL are anti-apoptotic, whereas other members of the Bcl-2 family, such as BAD or BAX are proapoptotic<sup>[33]</sup>. Bcl-2 has been shown to prevent the release of cytochrome C from mitochondria and hence activation of caspase 9, the initiator caspase<sup>[32]</sup>. Several kinases like JNK, p38<sup>[33]</sup> and cdc2/cyclin B kinase<sup>[34]</sup> have been noticed to phosphorylate/inactivate Bcl-2 as a physiological process during normal cell cycle progression or as a defense mechanism following the activation by various stimuli and stress. Phosphorylation/inactivation of Bcl-2 inactivates the antiapoptotic effect, which triggers the release of cytochrome C from the mitochondria leading to the activation of downstream caspases<sup>[35-37]</sup>.

Another important protein involved in apoptosis is



**Figure 1** Proposed model of induction of cell apoptosis and subsequent of cell atrophy by high glucose in kidney.

poly (ADP-Ribose) polymerase (PARP), a DNA repair enzyme that is cleaved by the downstream caspases. The essential role of PARP activation in diabetes induced by streptozotocin in adult male BALB/c mice<sup>[38]</sup>. PARP catalyzes the poly(ADP-ribosylation) of a variety of nuclear proteins with NAD substrate. Because it is activated by binding to DNA ends or strand breaks, an important feature of the cell in apoptosis, PARP was suggested to contribute to apoptosis by depleting the cell of NAD and ATP<sup>[39]</sup>. When PARP is cleaved into 89- and 24-kDa fragments that contain the active site and the DNA binding domain of the enzyme, respectively during drug induced apoptosis in a variety of cells<sup>[39]</sup>. Such cleavage essentially inactivates the enzyme by destroying its ability to respond to DNA strand breaks/fragmentation.

Proteases play a critical role in the initiation and execution of apoptosis. The caspases, a family of cysteine-dependent, aspartate-directed proteases, are prominent among apoptosis-associated molecules<sup>[40]</sup>. Activation of caspases cleaves a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of DNA repair machinery and a number of protein kinases. Caspase 3, a member of the caspase family plays a central role in the execution of the apoptotic program<sup>[41-43]</sup>. Oxidative stress mediated activation of caspase 3 has been shown to be a principle mediator of hyperglycemia induced proximal tubular apoptosis<sup>[5]</sup>. Caspase 3 is primarily responsible for the cleavage of PARP during cell death<sup>[41-45]</sup>. Recent published data show that high glucose and hyperglycemia induced cell apoptosis mainly in proximal tubular cells through regulation Bcl2/caspase/PARP pathway<sup>[46-49]</sup>. The sequence at which caspase 3 cleave PARP is very well conserved in the PARP protein from very distant species, indicating

the potential importance of PARP cleavage in apoptosis. Recent study from our lab showed the important role of tuberin/mTOR pathway in regulation of apoptosis<sup>[50]</sup>. We showed that induction of diabetes increased phosphorylation of tuberin in association with mTOR activation (measured by p70S6K phosphorylation), inactivation of Bcl-2, increased cytosolic cytochrome c expression, activation of caspase 3, and cleavage of PARP; insulin treatment prevented these changes. In addition, exposure of proximal tubular epithelial cells to high glucose increased phosphorylation of tuberin and p70S6K, phosphorylation of Bcl-2, expression of cytosolic cytochrome c, and caspase 3 activity. Moreover, high glucose induced translocation of the caspase substrate YY1 from the cytoplasm to the nucleus and enhanced cleavage of PARP. Cells treated with the mTOR inhibitor rapamycin resulted in reduce the number of apoptotic cells induced by high glucose<sup>[50]</sup>. This signaling cascade may play an important role in apoptosis induced by hyperglycemia during diabetic nephropathy. In summary, tubular apoptosis is one of the characteristic morphologic changes in human diabetic kidneys and tubular atrophy appears to be a better indicator of disease progression than glomerular pathology. A proposed model of induction of cell apoptosis and subsequent of cell atrophy by high glucose in kidney show in Figure 1. The mechanism by which hyperglycemia regulates apoptosis in renal tubular cells requires further study to provide the optimal management for diabetic complications.

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## Negative association between trunk fat, insulin resistance and skeleton in obese women

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### Abstract

**AIM:** To evaluate the potential interference of trunk fat (TF) mass on metabolic and skeletal metabolism.

**METHODS:** In this cross-sectional study, 340 obese women (mean age:  $44.8 \pm 14$  years; body mass index:  $36.0 \pm 5.9$  kg/m<sup>2</sup>) were included. Patients were evaluated for serum vitamin D, osteocalcin (OSCA), inflammatory markers, lipids, glucose and insulin (homeostasis model assessment of insulin resistance, HOMA-IR) levels, and hormones profile. Moreover, all patients underwent measurements of bone mineral density (BMD);

at lumbar and hip site) and body composition (lean mass, total and trunk fat mass) by dual-energy X-ray absorptiometry.

**RESULTS:** Data showed that: (1) high TF mass was inversely correlated with low BMD both at lumbar ( $P < 0.001$ ) and hip ( $P < 0.01$ ) sites and with serum vitamin D ( $P < 0.0005$ ), OSCA ( $P < 0.0001$ ) and insulin-like growth factor-1 (IGF-1;  $P < 0.0001$ ) levels; (2) a positive correlation was found between TF and HOMA-IR ( $P < 0.01$ ), fibrinogen ( $P < 0.0001$ ) and erythrocyte sedimentation rate ( $P < 0.0001$ ); (3) vitamin D levels were directly correlated with IGF-1 ( $P < 0.0005$ ), lumbar ( $P < 0.006$ ) and hip ( $P < 0.01$ ) BMD; and (4) inversely with HOMA-IR ( $P < 0.001$ ) and fibrinogen ( $P < 0.0005$ ). Multivariate analysis demonstrated that only vitamin D was independent of TF variable.

**CONCLUSION:** In obese women, TF negatively correlates with BMD independently from vitamin D levels. Reduced IGF-1 and increased inflammatory markers might be some important determinants that account for this relationship.

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**Key words:** Obesity; Skeleton; Vitamin D; Osteocalcin; Insulin resistance; Trunk fat; Inflammation

**Core tip:** Recent studies have shown that high fat mass content might be a risk factor for osteoporosis and fragility fractures. We evaluated obese women for vitamin D, osteocalcin, inflammatory markers, metabolic and hormones profile, bone mineral density (BMD) and body composition by dual-energy X-ray absorptiometry. Our results show that in obese women trunk fat negatively correlates with BMD independently from vitamin D levels, likely as consequence of reduced insulin-like growth factor-1 and increased inflammatory markers.

These data indicate that obesity cannot be considered a protective factor for osteoporosis and suggest that obese postmenopausal women should be investigated for possible alterations of skeletal metabolism.

Greco EA, Francomano D, Fornari R, Marocco C, Lubrano C, Papa V, Wannenes F, Di Luigi L, Donini LM, Lenzi A, Aversa A, Migliaccio S. Negative association between trunk fat, insulin resistance and skeleton in obese women. *World J Diabetes* 2013; 4(2): 31-39 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i2/31.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i2.31>

## INTRODUCTION

Obesity and osteoporosis are two important global health problems with an increasing prevalence and high impact on both mortality and morbidity<sup>[1-4]</sup>. Interestingly, during the last decades both diseases have become a major health threat around the world, with age and female status increasing the risk of developing both obesity and osteoporosis<sup>[1-4]</sup>.

Obesity has been considered a protection factor against the development of bone loss and osteoporosis, likely for increased androgen aromatization to estrogens in postmenopausal obese women<sup>[5,6]</sup>. Additionally, mechanical loading appears to stimulate bone formation by decreasing apoptosis and increasing proliferation and differentiation of both osteoblasts and osteocytes<sup>[7]</sup> by an activation of the intracellular signalling Wnt/ $\beta$ -catenin<sup>[8-10]</sup>. Therefore, the mechanical loading conferred by body weight justified the assumption of a protective role of obesity in the prevention of osteoporosis<sup>[5]</sup>.

More recently, however, the belief that obesity is protective against osteoporosis has been questioned. In fact, epidemiologic and clinical studies have suggested that high level of fat mass might be a risk factor for osteoporosis and fragility fractures<sup>[11-13]</sup>. Indeed, adipose tissue not only stores excess triacylglycerols, but functions as an endocrine organ by releasing several adipokines, which appear to modulate glucose and lipid metabolism, inflammation, appetite and insulin resistance<sup>[14-16]</sup>. Additionally, the physiological relevance of adipose tissue for skeletal health likely resides in the role that some of these adipokines, such as interleukin (IL)-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), might play by interfering with bone cells homeostasis<sup>[17-20]</sup>. Moreover, bone has started to be considered an endocrine organ itself affecting both body weight control and glucose homeostasis through the action of bone-derived factors such as osteocalcin and osteopontin<sup>[21,22]</sup>. This cross-talk between fat and bone seems to play an important role as homeostatic feedback system in which adipokines and molecules secreted by bone cells might represent the link of an active and functional bone-adipose-glucose axis<sup>[23-25]</sup>, by mechanism(s) not fully clarified yet.

Recent evidences suggest that obesity is also associated with a chronic low-grade inflammation as depicted by increased plasma levels of C-reactive protein (CRP), pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, and osteopontin<sup>[26-30]</sup>. Few reports also depict an association between obesity and circulating low levels of vitamin D<sup>[31-33]</sup>. Nevertheless to date, few and conflicting data exist about possible correlation among vitamin D, total intact osteocalcin (OSCA), inflammatory markers<sup>[32-35]</sup> and bone mineral density (BMD) in obese women.

Since our group has recently demonstrated that a sub-population of adult obese subjects had significant skeletal alterations, and that different levels of adiposity could differently affect skeletal health<sup>[12]</sup>, the aim of the present study was to evaluate potential detrimental correlations between obesity, vitamin D levels, inflammation and BMD in obese female subjects.

## MATERIALS AND METHODS

### Patients

In this study, 340 women [mean age:  $44.8 \pm 14$  years; mean body mass index (BMI):  $36.0 \pm 5.9$  kg/m<sup>2</sup>] were selected from a cohort of patients admitted to the day hospital of Department of Experimental Medicine, Section of Medical Pathophysiology, Endocrinology and Nutrition, Policlinico Umberto I, Sapienza University of Rome, for the diagnosis and therapy of obesity.

The study received the approval of the Internal Review Board of our Institution. Exclusion criteria were chronic medical conditions or the use of medications affecting bone metabolism, hormonal and nutritional status, vitamin D supplementation, recent weight loss, and prior bariatric surgery interventions. Patients underwent complete medical history and clinical examination. Anthropometric measurements included weight and height; body weight was measured as the subjects were fasting overnight and wearing underwear. BMI was calculated as weight (kg)/height (m<sup>2</sup>).

### Biochemical analysis

Hormones, lipid profile, glucose, insulin levels, fibrinogen, CRP, calciotropic hormones were evaluated. Additionally, OSCA, the well known most abundant non-collagenic bone matrix protein, marker of bone turnover, was measured by standard methods. Measurements of glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides and fibrinogen concentrations were assessed by standard immune-enzymatic methods, while insulin and vitamin D levels were measured by radioimmunoassay. Serum parathyroid hormone was measured by a two-site immunoradiometric assay, and CRP circulating levels were measured by latex agglutination. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting plasma insulin and glucose levels using the formula:  $\text{insulin} \times \text{glucose} / 22.5$  (mU/L  $\times$  mmol/L).

**Table 1** Baseline demographics of the study population (mean  $\pm$  SD)

Characteristics	n = 340
Mean age (yr)	44.8 $\pm$ 14
BMI (kg/m <sup>2</sup> )	36 $\pm$ 5.9
Total cholesterol (mg/dL)	196 $\pm$ 41
HDL-cholesterol (mg/dL)	49 $\pm$ 11
Triglycerides (mg/dL)	120 $\pm$ 68
HOMA-IR	4.7 $\pm$ 3
SHBG (nmol/L)	42 $\pm$ 58.5
PTH (pg/mL)	45 $\pm$ 21
Leptin (nmol/L)	75 $\pm$ 30
Vitamin D	20.5 $\pm$ 9.8
17 $\beta$ -estradiol	70 $\pm$ 54

BMI: Body mass index; HOMA-IR: Homeostasis model assessment of insulin resistance; Vitamin D: 25-hydroxyvitamin D; PTH: Parathyroid hormone; HDL: High-density lipoprotein; SHBG: Sex hormone binding globulin.

**Dual-energy-X-ray absorptiometry measurement**

Body fat mass, fat-free mass (kg) and both lumbar and femoral BMD were measured by dual-energy-X-ray absorptiometry (DEXA) (Hologic 4500 RDR), with coefficient of variation of < 1% for bone density and < 1.5% for fat mass<sup>[12]</sup>. Amount of trunk fat mass was distinguished from peripheral and appendicular fat mass as a measure of abdominal adiposity. In particular, trunk fat was defined as the adipose tissue localized within the region below the chin, delineated by vertical lines within the left and right glenoid fossae bordering laterally to the ribs, and by the oblique lines that cross the femoral necks and converge below the pubic symphysis.

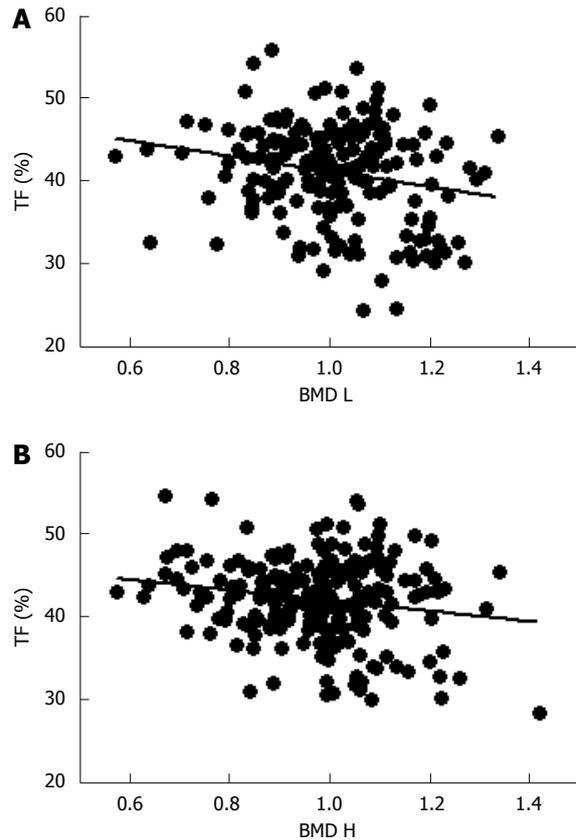
**Statistical analysis**

Results are expressed as mean  $\pm$  SD and compared by means of analysis of variance for repeated measures. Pearson correlations were used to examine associations between variables, and multiple regression analyses were used to determine the influence of TF and vitamin D on the different variables. *P* < 0.05 defined differences statistically significant as described elsewhere<sup>[12]</sup>. Multivariate linear regression analysis was carried out to identify the independent relations of TF by including the parameters which were related with TF on bivariate analysis by using SPSS/4.0 (SPSS, Chicago, IL, United States) and SAS/6.4 (SAS Institute, Cary, NC, United States).

**RESULTS**

A total of 340 obese women were observed and clinical characteristics are shown in Table 1, which shows the presence of obesity.

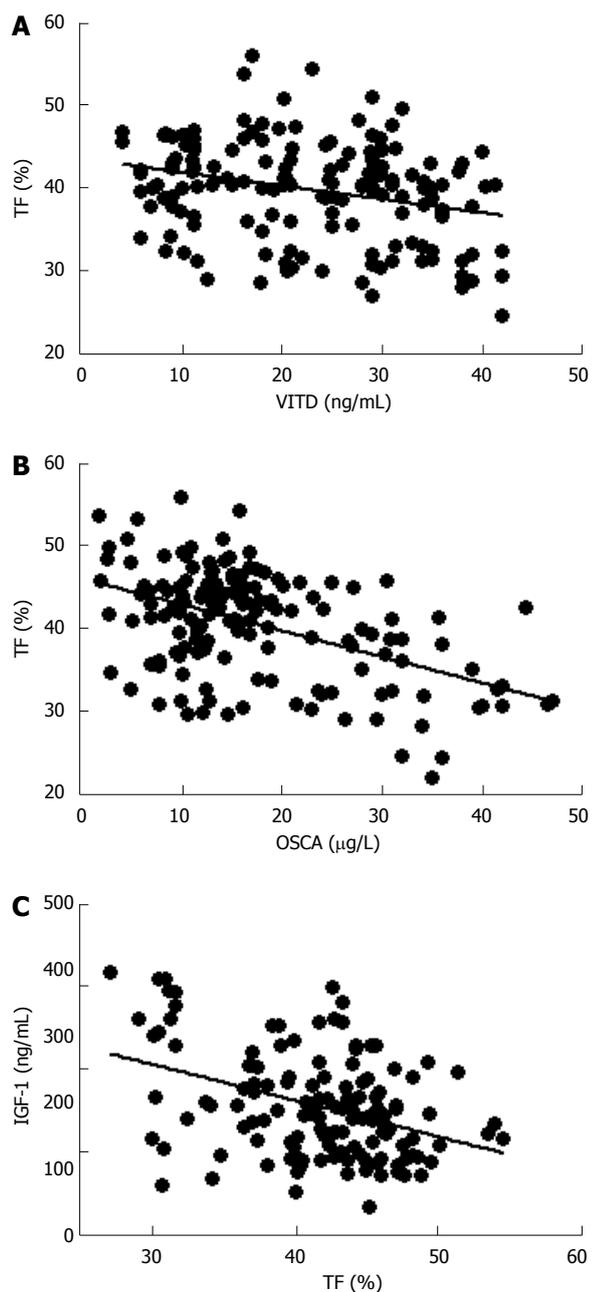
Initial analysis of the obese subjects showed a positive correlation between body weight and lumbar and femoral BMD (data not shown) as previously reported in the literature<sup>[6,36]</sup>. However, further evaluation to characterize potential relationship between fat tissue distribution and skeleton alteration showed a significant inverse relation-



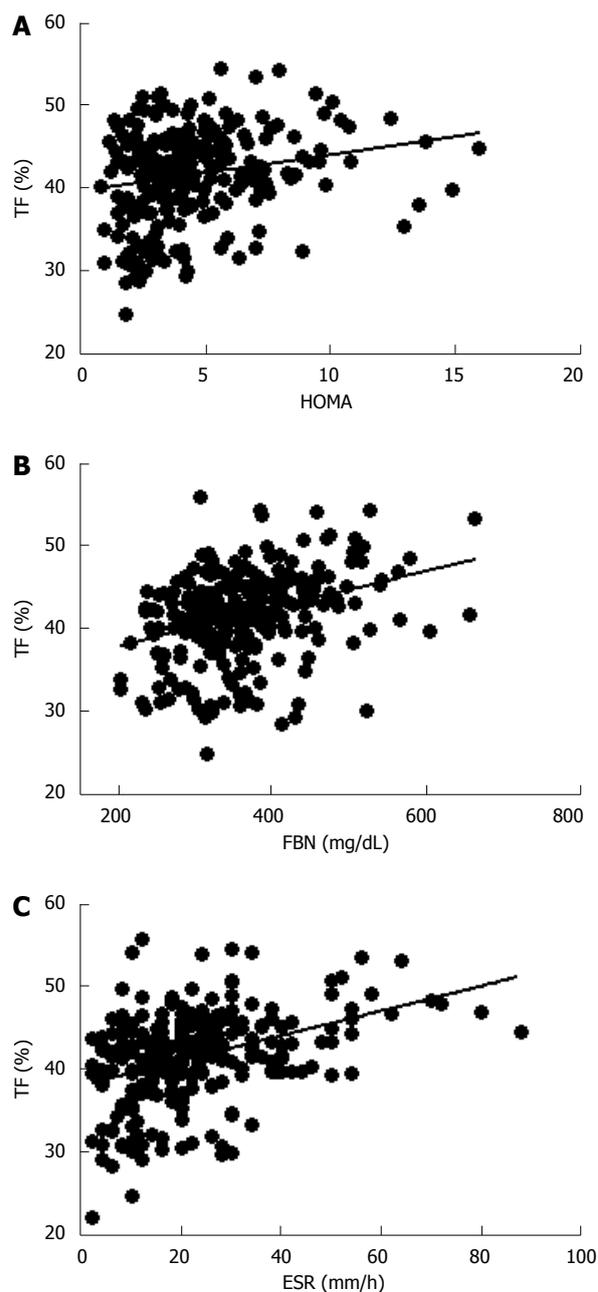
**Figure 1** Correlation between trunk fat and bone mineral density at both lumbar and femoral sites. A: Trunk fat (TF) percentage and bone mineral density at the lumbar (BMD L; *r* = -0.22, *P* < 0.001); B: Bone mineral density at the hip (BMD H, *r* = -0.22, *P* < 0.01).

ship between TF and BMD at both lumbar and femoral sites (Figure 1), suggesting a detrimental role of abdominal fat on skeletal mass.

Further evaluation of these obese women demonstrated that vitamin D levels were significant lower than normal range, and these values were inversely correlated to either BMI (data not shown) and trunk adiposity (Figure 2A). Additionally, to correlate obesity with alteration of bone markers, OSCA levels were evaluated in these female subjects and correlated to adipose tissue. As shown in Figure 2B, OSCA levels were inversely correlated with TF mass suggesting that adipose tissue might have a detrimental effect on this specific osteoblast-specific hormone. Also insulin-like growth factor-1 (IGF-1) serum levels were inversely correlated with TF (Figure 2C). Moreover a strong direct correlation was found between vitamin D and OSCA levels (data not shown). Further, a direct relationship between TF and HOMA-IR index (Figure 3A), and inflammatory markers such as fibrinogen (Figure 3B) and erythrocyte sedimentation rate (Figure 3C) was found in these obese adult female subjects indicating, as suggested by others<sup>[21,22]</sup>, a potential role of TF in glucose homeostasis. Analysis carried out to investigate possible relationship between IGF-1 levels and vitamin D status showed a strong direct relationship (Figure 4A). Also, vitamin D levels were directly corre-



**Figure 2** Inverse relationship between trunk fat percentage and vitamin D (A;  $r = -0.27$ ,  $P < 0.0005$ ), osteocalcin (B;  $r = -0.49$ ,  $P < 0.0001$ ) and insulin-like growth factor-1 (C;  $r = -0.31$ ,  $P < 0.0001$ ) plasma levels in obese women. VITD: Vitamin D; OSCA: Osteocalcin; IGF-1: Insulin-like growth factor-1; TF: Trunk fat.

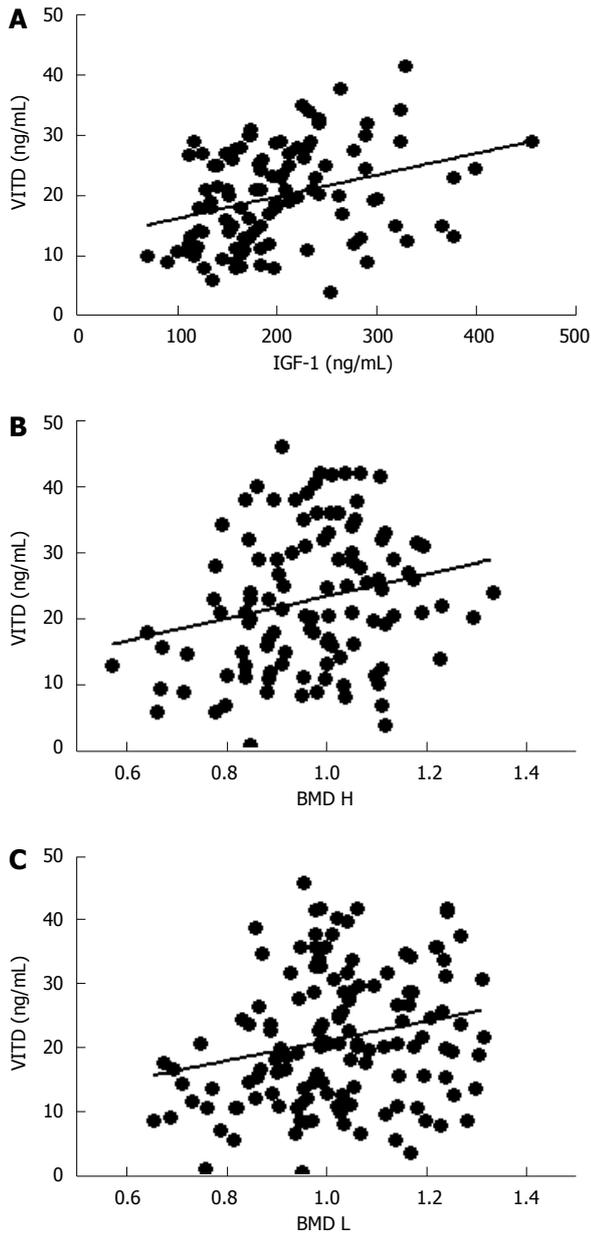


**Figure 3** Direct relationship between trunk fat percentage, homeostasis model assessment index (A;  $r = 0.18$ ,  $P < 0.01$ ), fibrinogen (B;  $r = 0.44$ ,  $P < 0.0001$ ) and erythrocyte sedimentation rate (C;  $r = 0.29$ ,  $P < 0.0001$ ) in obese women. HOMA: Homeostasis model assessment; FBN: Fibrinogen; ESR: Erythrocyte sedimentation rate; TF: Trunk fat.

lated with BMD at the femoral (Figure 4B) and lumbar (Figure 4C) sites and inversely correlated with HOMA-IR (Figure 5A) and fibrinogen levels (Figure 5B). Since it is known that obesity is associated with a low-grade inflammation<sup>[37,38]</sup>, specific markers were also investigated. As expected, inflammatory markers were significantly elevated in obese women (Table 2) with a strong correlation with degree of obesity. Multivariate analysis demonstrated that only vitamin D was the only parameter that resulted to be independent from TF (Table 3).

## DISCUSSION

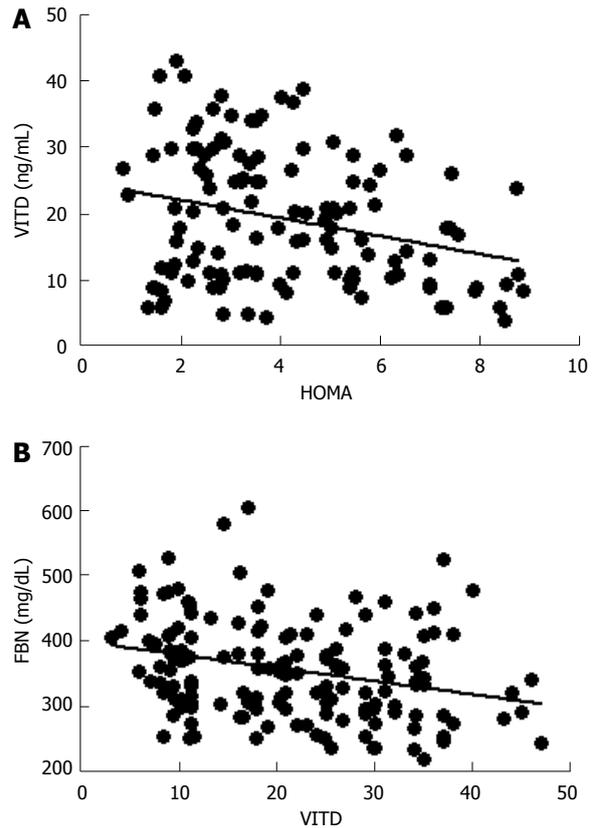
The results presented herein show for the first time that in obese women, the amount of TF is negatively correlated with BMD, vitamin D, osteocalcin and IGF-1 levels, whereas it is directly correlated with insulin insensitivity and inflammation markers. Also, vitamin D status was directly correlated with IGF-1 levels and multivariate analysis showed that it was the only parameter that was independently associated with TF. This represents a novel



**Figure 4** Direct relationship between vitamin D, insulin-like growth factor-1 (A;  $r = 0.32$ ,  $P < 0.0005$ ), hip (B;  $r = 0.23$ ,  $P < 0.01$ ) and lumbar bone mineral density (C;  $r = 0.19$ ,  $P < 0.005$ ) in obese women. VITD: Vitamin D; IGF-1: Insulin-like growth factor-1; BMD H: Bone mineral density at the hip; BMD L: Bone mineral density at the lumbar; TF: Trunk fat.

finding in obese women, suggesting that vitamin D and IGF-1 levels might be considered a sensitive predictor and indicator of skeletal health, as bone mineral density alteration itself.

Fat tissue is present throughout the body and, in cases of obesity, can cover up to 50% or more of the entire body mass. White adipose tissue (WAT) is the most abundant form, found in both subcutaneous and intra-abdominal regions. WAT was first regarded only as an energy reservoir, however it is now well recognized as an endocrine organ due to its secretion of circulating adipokines and pro-inflammatory factors<sup>[14-20]</sup>. Obesity, defined as an abundance of WAT, has always been depicted as a



**Figure 5** Inverse relationship between vitamin D levels with homeostasis model assessment (A;  $r = -0.27$ ,  $P < 0.001$ ) and with fibrinogen (B;  $r = -0.28$ ,  $P < 0.0005$ ). VITD: Vitamin D; HOMA: Homeostasis model assessment; FBN: Fibrinogen.

protective factor against the development of bone loss and osteoporosis<sup>[5,6]</sup>, nevertheless several groups, including ours<sup>[11-13]</sup>, have recently demonstrated that high amounts of adipose tissue accumulation might not be considered a protective factor against the development of osteoporosis and fracture risk.

Thus, the main objective of our study was to evaluate the relationship between obesity ( $BMI > 30 \text{ kg/m}^2$ ) and BMD modifications. Interestingly, while BMD was correlated to BMI, body weight appeared to be a protective factor against low bone mass (data not shown), as previously reported in the literature<sup>[5,6]</sup>, which led to claim a protective role of obesity against bone loss and osteoporosis. However, data were re-analyzed to evaluate potential detrimental role of body fat distribution on skeletal health. This evaluation demonstrated that higher level of TF correlated with lower bone mass, strongly suggesting that BMI might not be considered the unique parameter to evaluate potential detrimental effect of fat tissue as risk factors for cardiovascular, metabolic or skeletal disorders<sup>[1-2,23,39-42]</sup>. Indeed, recent data indicate that TF might correlate with skeletal damages in young population as well<sup>[43]</sup>.

Moreover, although obese subjects have greater calories intake than subjects with normal body weight, they often show nutritional deficiencies or alterations in hormonal or metabolic parameters. For instance, obese women show very low concentrations of vitamin D, as described

**Table 2 Biochemical and hormonal characteristics of the study population according to different body mass index**

	BMI < 30 kg/m <sup>2</sup> (n = 80)	BMI 30-35 kg/m <sup>2</sup> (n = 100)	BMI 35-40 kg/m <sup>2</sup> (n = 80)	BMI > 40 kg/m <sup>2</sup> (n = 80)
BMI	27 ± 1.2	32.5 ± 1 <sup>b</sup>	37 ± 1.5 <sup>b</sup>	44 ± 2 <sup>b</sup>
Mean age (yr)	46.5 ± 15	45 ± 14	46 ± 13	43 ± 14
Total-cholesterol (mg/dL)	199 ± 54	198 ± 41	204 ± 40	190 ± 32
HDL-cholesterol (mg/dL)	52 ± 13	50 ± 11	50 ± 10	46 ± 10
Triglycerides (mg/dL)	114 ± 79	117 ± 61	130 ± 72	122 ± 68
Fibrinogen (mg/dL)	346 ± 102	341 ± 63	368 ± 85	421 ± 86 <sup>b</sup>
C-reactive protein (ng/mL)	2 ± 0.9	2.8 ± 0.9	5.0 ± 1.4 <sup>b</sup>	5.5 ± 2.1 <sup>b</sup>
HOMA-IR	2.8 ± 0.9	3.1 ± 0.8	5.0 ± 2.6 <sup>b</sup>	6.4 ± 2.2 <sup>b</sup>
PTH (pg/mL)	40 ± 15	42 ± 20	46 ± 23	48 ± 19
Vitamin D	26 ± 9	20 ± 10 <sup>b</sup>	16 ± 8 <sup>b</sup>	15 ± 10 <sup>b</sup>

<sup>b</sup>P < 0.01 vs BMI < 30 kg/m<sup>2</sup>. BMI: Body mass index; HOMA-IR: Homeostasis model assessment of insulin resistance; Vitamin D: 25-hydroxyvitamin D; PTH: Parathyroid hormone; HDL: High-density lipoprotein.

**Table 3 Multivariate analysis showing that vitamin D is the only parameter that is independently associated with trunk fat percentage**

Model	Unstandardized coefficients <sup>1</sup>		Standardized coefficients <sup>1</sup>		
	B	SE	Beta	t	P value
1 (constant)	52.054	8.498		6.125	0.000
Lumbar BMD	6.570	8.121	0.167	0.809	0.437
Hip BMD	-9.971	7.687	-0.259	-1.029	0.224
Vitamin D	-0.359	0.077	-1.029	-4.666	0.001
Osteocalcin	0.134	0.111	0.255	1.208	0.255
IGF-1	-0.002	0.014	-0.022	-0.114	0.916

<sup>1</sup>Dependent variable of trunk fat. IGF-1: Insulin-like growth factor-1; BMD: Bone mineral density.

by others<sup>[33-35]</sup>, as well as the osteoblast-produced OSCA, which were inversely correlated to TF mass, suggesting that alteration of biochemical and hormonal parameters might be an indicator of skeletal damage and decreased density as diagnosed by DEXA. As previously shown by others, we also observed an inverse relationship between vitamin D and BMI, likely due to the amount of adipose tissue, which, in individuals who are not obese, is inversely associated with its blood concentrations<sup>[44,45]</sup>. As described in the literature, we confirmed a positive correlation between low vitamin D circulating levels and low BMD also in obese women, but we found a new direct relationship between vitamin D, IGF-1 and TF; this highlights the fact that bone tissue might indeed play a pivotal role in the recently described feedback among fat, bone and glucose metabolism<sup>[25,46,47]</sup>.

In the last years, potential association between obesity, cardiovascular and metabolic diseases such osteoporosis, has been actively investigated and common pathogenic links have been proposed since all are influenced by genetic and environmental factors, or by the interaction of such factors. Aging is associated with these chronic diseases and with a high incidence of bone loss and bone marrow adiposity; in turn, bone remodeling and adiposity are regulated through a complex concert of adipo-

kines and hormone interactions. Indeed, adipocytes and osteoblasts derive from a common progenitor cell, that is the mesenchymal stem cell<sup>[23,48]</sup>, and several potential mechanisms have been proposed to explain the complex relationship between adipose and bone tissues<sup>[47-50]</sup>.

Adipose tissue was long viewed as a passive energy reservoir, but since the discovery of leptin, and other adipose tissue-derived factors<sup>[28,49,50]</sup>, fat has been considered an active endocrine organ. Indeed, it (TF) secretes inflammatory cytokines, such as IL-6 and TNF- $\alpha$ <sup>[51]</sup>, which appear to play a pivotal role in the maintenance of the low-grade inflammatory status of obesity, leading to the development of adverse metabolic and cardiovascular consequences and, likely, contributing to the detrimental effect of fat tissue on the skeleton<sup>[20]</sup>.

Evidences suggest that an inflammatory status might be involved in the pathogenesis of osteoporosis promoting osteoclasts differentiation and activity and maintaining an altered bone remodeling<sup>[52-57]</sup>. Recently, CRP, an inflammatory marker, has been identified as an independent risk factor for cardiovascular events in healthy postmenopausal women<sup>[52-57]</sup> and high serum levels of CRP are also associated with lower BMD, higher levels of bone turnover markers and, more recently, greater risk of fracture<sup>[52-57]</sup>, further suggesting a role of inflammation in bone loss pathogenesis. At the present time it is unknown whether CRP plays a pivotal role as mediator of bone loss similarly to its role in atherosclerosis<sup>[57]</sup> or whether is only a marker of systemic inflammation, linked to bone health alterations<sup>[53]</sup>. In the present study we found an association between inflammatory markers, *i.e.*, of erythrocyte sedimentation rate and fibrinogen, vitamin D levels and insulin resistance, thus suggesting that a higher degree of inflammation might be in part responsible for deterioration of bone health.

Finally, we also found a negative correlation between high degree of obesity and IGF-1 level (inversely related to lean mass, data not shown) which also correlated with lower BMD in obese women. The importance of this factor in bone tissue homeostasis is well known<sup>[57]</sup> both during infancy and adulthood, but our data further in-

dicate that a complex metabolic and hormonal pattern alteration exist in obesity which is linked to bone homeostasis alteration.

In conclusion, our data show that TF plays a detrimental role in skeletal metabolism both in term of low BMD, bone markers and systemic factors influencing skeletal tissue. Finally, alteration of vitamin D levels, and inflammation status, in association with low OSCA, altered insulin sensitivity might indicate the existence of an important interplay between bone tissue, energy metabolism and inflammations, which might suggest a common pathogenic mechanism in the development of metabolic, cardiovascular and skeletal diseases. Further studies are however needed to fully clarify and characterize the mechanism(s) underlying the role of trunk fat in the development effect of chronic diseases, such diabetes, cardiovascular disease and osteoporosis.

## COMMENTS

### Background

Obesity and osteoporosis are two important global health problems with an increasing prevalence and high impact on both mortality and morbidity. The belief that obesity is protective against osteoporosis has been questioned. In fact, epidemiologic and clinical studies have suggested that high level of fat mass might be a risk factor for osteoporosis and fragility fractures

### Research frontiers

Recent evidences suggest that obesity is also associated with a chronic low-grade inflammation as depicted by increased plasma levels of C-reactive protein, pro-inflammatory cytokines. Few reports also depict an association between obesity and circulating low levels of vitamin D.

### Innovations and breakthroughs

The results presented herein show for the first time that in obese women, the amount of trunk fat (TF) is negatively correlated with bone mineral density (BMD), vitamin D, osteocalcin and insulin-like growth factor-1 (IGF-1) levels, whereas it is directly correlated with insulin insensitivity and inflammation markers.

### Applications

This data show that TF plays a detrimental role in skeletal metabolism both in term of low BMD, bone markers and systemic factors influencing skeletal tissue.

### Peer review

This is an interesting article on the associations of trunk fat with inflammation biomarkers, IGF-1 and bone density in severe obese women.

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## Parental transmission of type 2 diabetes mellitus in a highly endogamous population

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### Abstract

**AIM:** To determine the parental transmission of diabetes mellitus (DM) and evaluate its influence on the clinical characteristics.

**METHODS:** This was a cross sectional study. The survey was carried out in urban and semi-urban primary health care centers. Of the 2400 registered with diagnosed diabetes, 1980 agreed and gave their consent to take part in this study, thus giving a response rate of 82.5%. Face to face interviews were conducted using a structured questionnaire followed by laboratory tests. DM was defined according to the World Health Organization expert group. A trained nurse performed

physical examinations and measurements.

**RESULTS:** Of the study population, 72.9% reported a family history of DM. Family history of DM was significantly higher in females (54.2%;  $P = 0.04$ ) and in the age group below 30 years (24%;  $P < 0.001$ ). The prevalence of diabetes was higher among patients with a diabetic mother (25.4% vs 22.1%) and maternal aunts/uncles (31.2% vs 22.2%) compared to patients with a diabetic father and paternal aunts/uncles. Family history of DM was higher in patients of consanguineous parents (38.5%) than those of non-consanguineous parents (30.2%). The development of type 2 diabetes mellitus (T2DM) complications was higher in patients with either a paternal or maternal history of DM than in those without. No significant difference was observed in the metabolic characteristics of patients with/without family history of DM except for hypertension. Complications were higher in diabetic patients with a family history of DM.

**CONCLUSION:** The present study found a significant maternal effect in transmission of T2DM. Family history is associated with the increased incidence of diabetes.

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**Key words:** Diabetes mellitus; Family history; Parental transmission; Genetic disorders; Consanguinity; Maternal transmission

**Core tip:** Diabetes is a disease that has a strong clustering in families and has a genetic component. Family history is a well-known risk factor for developing of type 2 diabetes mellitus (T2DM). The present study found a significant maternal effect in transmission of T2DM. Family history is associated with the increased incidence of diabetes.

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## INTRODUCTION

Diabetes is a multifactorial disease that involves complex interactions between genes, environment and health behavior. Type 2 diabetes mellitus (T2DM) is a common metabolic disorder, characterized by hyperglycemia caused by impaired glucose homeostasis, and represents a serious public health problem in many developed countries<sup>[1]</sup>. Current studies have revealed a definite global increase in the incidence and prevalence of diabetes. In 2000, 171 million people were estimated to be diabetic worldwide, which is projected to rise to 366 million cases in the year 2030<sup>[2]</sup>. It is the fourth or fifth leading cause of death in most developed countries<sup>[1]</sup>. Given the growing rate of diabetes and its far reaching societal and economic consequences, prevention of diabetes among people at high risk is a public health issue of clinical importance.

Diabetes is a disease that has a strong clustering in families and has a genetic component. It has been widely reported that the occurrence of T2DM is triggered by a genetic susceptibility and familial aggregation in several populations<sup>[3,4]</sup>. Family history is a well-known risk factor for the developing of T2DM. It was estimated that risk for diagnosed T2DM increases approximately two to four fold when one or both parents are affected<sup>[5]</sup>. Almost 25% to 33% of all T2DM patients have family members with diabetes. Having a first degree relative with the disease poses a 40% risk of developing diabetes<sup>[6]</sup>. T2DM patients are more likely to have diabetic mothers than diabetic fathers. The existence of excess maternal transmission of T2DM in offspring of affected mothers than affected fathers is currently debated<sup>[7]</sup>. Family history reflects both inherited genetic susceptibilities and shared environments which include cultural factors<sup>[8]</sup>. Thus, family history of diabetes may be a useful tool to identify individuals at increased risk of the disease and target behavior modifications that could potentially delay disease onset and improve health outcomes.

It was reported that several genetic disorders, congenital malformations and reproductive wastage are more frequent in consanguineous marriages<sup>[9]</sup>. A previous study by Bener *et al*<sup>[10]</sup> showed significant increase in the prevalence of common adult diseases in a population with a high rate of consanguinity. The incidence of consanguinity (51%) is relatively high in the State of Qatar with first cousin marriage predominantly comprising 26.7% of all marriages.

In Qatar, it was reported that diabetes is on the rise and if proper intervention and preventive strategies were not adopted, the epidemic of diabetes will prove fatal. The upcoming epidemic and projected increase in the prevalence of diabetes over the next two decades emphasize the importance of early detection<sup>[11]</sup> of diabetes in the population. Few studies have documented the prevalence of T2DM and its complications in the

population of Qatar<sup>[12,13]</sup>. To the best of our knowledge, the patterns of familial transmission of T2DM in Qatar have not been studied so far. The significance of maternal or paternal inheritance in diabetes has been a matter of controversy and difference in various populations and races. Hence, this is the first cross-sectional survey of the Arab population in Qatar to determine the influence of familial history of T2DM in the offspring and evaluate its influence on the clinical characteristics of this disease.

## MATERIALS AND METHODS

This is a cross-sectional study which was conducted among diabetic patients registered in diabetic clinics of primary health care (PHC) centers of the Supreme Council of Health. The diabetes care is organized in most of the PHC centers. During the study period from January 2010 to January 2011, the study included T2DM patients registered in these diabetic clinics who were taking oral hypoglycemic drugs. In this study, multistage stratified cluster sampling was employed using the administrative divisions of the PHC in Qatar. Target population of each PHC is approximately equal. Stratification was done to obtain a representative sample of target population, with equal proportions from both urban and semi urban areas. The sample size was statistically calculated based on 17% prevalence rate of diabetes in Qatar<sup>[13]</sup>, with 1% level of significance and assuming 2% bound on error of estimation, giving a minimum sample size of 2400 subjects for this study. Of the 2400 patients approached from different PHC centers (10 centers from an urban area and 2 centers from a semi-urban area), 1980 agreed to participate and gave verbal consent to take part in this study (82.5%). Also, any patients with incomplete laboratory values in the medical records were excluded from the study. The study was approved by the Hamad Medical Corporation prior to commencing data collection.

### Questionnaire

We developed a structured questionnaire consisting of questions relating to socio-demographic data, family history of diabetes mellitus (DM), lab investigations and complications. The first part included information about socio-demographic characteristics, including age, sex, marital status, education level, occupation, height, weight, blood pressure and parental consanguinity. The second section collected information about family history of DM with family relations and complications after the onset of diabetes. The third section included items about laboratory investigations such as blood glucose, glycated hemoglobin, high-density lipoprotein/low-density lipoprotein cholesterol levels, triglyceride, urea, creatinine, bilirubin, albumin *etc.* Necessary corrections and modifications were made in the questionnaire after the pilot study. Content validity, face validity and reliability of the questionnaire were tested using 50 subjects. These tests demonstrated a high level of validity and high degree of repeatability ( $\kappa = 0.84$ )<sup>[14]</sup>. Family physicians and research nurses reviewed the medical files of diabetic patients in PHC and recorded all lab

investigation measurements from their files.

### Physical examination and other measurements

A trained nurse performed physical examinations and measurements. In order to measure height (m), participants were asked to stand in bare feet while maintaining a straight posture on a height scale (SECA, Germany). Similarly, weight (kg) was measured using the same scale with light clothing and bare feet. Body mass index was calculated as the ratio of weight (kg) to the square of height (m).

Hypertension was defined as per World Health Organization (WHO) standardized criteria, "systolic blood pressure (SBP)  $\geq$  140 mmHg or diastolic blood pressure (DBP)  $\geq$  90 mmHg or using anti-hypertensive medication"<sup>[15]</sup>. In order to measure blood pressure, subjects were asked to sit and rest for at least 10-15 min. Two readings of SBP and DBP were taken from his/her left arm at heart level while using standard zero mercury sphygmomanometer. An average of both readings for SBP and DBP was obtained.

### Laboratory measurements

Study participants with a history of T2DM and currently taking oral anti-diabetic medications were considered to have DM. DM was defined as per the WHO expert group<sup>[16]</sup>, *i.e.*, fasting venous blood glucose (FBS) concentration  $\geq$  7.0 mmol/L and/or 2 h post-oral glucose tolerance test (OGTT) venous blood glucose concentration  $\geq$  11.1 mmol/L. FBS was measured by glucose meter among all the participants and those with FBS < 7 mmol/L were further tested by an OGTT. In order to conduct OGTT, participants were asked to drink 75 g anhydrous glucose dissolved in 250 mL water within the space of 5 min. Samples were processed within 30 min of collection and the above laboratory tests were measured. Subjects with impaired FBS (venous blood glucose concentration for 5.6-6.9 mmol/L) or impaired OGTT (2 h post-OGTT venous blood glucose level of 7.8-11.0 mmol/L) were labeled as pre-diabetes. Glycosylated hemoglobin was analyzed using a high-performance liquid chromatography method with a range > 6.5% defined as "unsatisfactory" metabolic control.

### Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS, version 19) software. Standard descriptive statistical analysis was performed. Student *t* test was used to ascertain the significance of differences between mean values of two continuous variables and one way analysis of variance was used to find the differences between continuous variables among more than two groups. Differences between categorical variables were tested through Pearson  $\chi^2$  or Fisher's exact test when the assumptions for  $\chi^2$  test were not fulfilled. Two sided *P* value of less than 5% was considered as significant.

## RESULTS

Table 1 shows the socio-demographic characteristics of

**Table 1** Socio-demographic characteristics of patients with/without a family history of diabetes mellitus *n* (%)

	With family history of DM <sup>1</sup> ( <i>n</i> = 1444)	Without family history of DM ( <i>n</i> = 536)	<i>P</i> value
Age (yr)			< 0.001
18-30	346 (24.0)	72 (13.4)	
30-39	280 (19.4)	66 (12.3)	
40-49	258 (17.9)	132 (24.6)	
50-59	400 (27.7)	194 (36.2)	
$\geq$ 60	160 (11.1)	72 (13.4)	
Gender			0.041
Male	661 (45.8)	273 (50.9)	
Female	783 (54.2)	263 (49.1)	
Nationality			0.020
Qatari	838 (58.0)	342 (63.8)	
Other Arabs	606 (42.0)	194 (36.2)	
Educational level			0.154
Illiterate	240 (16.6)	71 (13.2)	
Elementary	267 (18.5)	114 (21.3)	
Intermediate	305 (21.1)	99 (18.5)	
Secondary	381 (26.4)	152 (28.4)	
University	251 (17.4)	100 (18.7)	
Occupation			0.134
Housewife	370 (25.6)	128 (23.9)	
Sedentary	356 (24.7)	162 (30.2)	
Professional	249 (17.2)	97 (18.1)	
Manual	153 (10.6)	53 (9.9)	
Businessmen	127 (8.8)	39 (7.3)	
Army/police clerk	189 (13.1)	57 (10.6)	
Monthly household income (QR)			0.589
< 5000	93 (6.4)	39 (7.3)	
5000-10 000	470 (32.5)	175 (32.6)	
10 000-15 000	510 (35.3)	200 (37.3)	
> 15 000	371 (25.8)	122 (22.8)	
Consanguinity			0.001
Yes	556 (38.5)	162 (30.2)	
No	888 (61.5)	374 (69.8)	

<sup>1</sup>Up to the third generation, two sided *P* values based on Pearson  $\chi^2$  test. DM: Diabetes mellitus; QR: Qatar Riyal.

patients with/without a family history of DM. Family history of DM was significantly higher in female patients (54.2%; *P* = 0.041), Qatari nationals (58%; *P* = 0.020) and in the age group below 30 years (24%; *P* < 0.001). Consanguinity was significantly higher in diabetic patients with family history of DM (38.5% *vs* 30.2%; *P* = 0.001) compared to those without family history of DM.

Table 2 reveals the familial history of diabetes mellitus among diabetic patients. Of the total study population, 72.9% reported a family history of DM. The prevalence of DM in father, mother, brother and sister was 22.1%, 25.4%, 14.2% and 9.3% respectively. In 2<sup>nd</sup> degree relatives for uncles and aunts, a positive history of T2DM was more common among maternal aunts/uncles than in paternal aunts/uncles (31.2% *vs* 22.2%). On the maternal side, 83.7% of the diabetic patients have an affected mother (25.4%) and at least one relative (58.3%), compared to only 67.3% of diabetic patients with an affected father (22.1%) and one family member (45.2%) on the paternal side.

Table 3 gives physical, metabolic characteristics and complications among diabetic patients according to the

**Table 2** Familial history of diabetes mellitus among diabetic patients *n* (%)

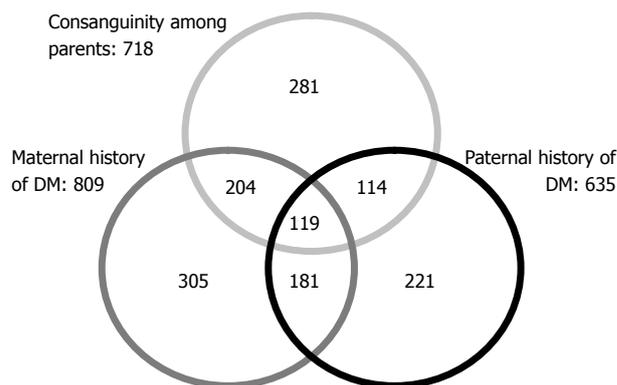
	<i>n</i> = 1980
Family history of diabetes <sup>1</sup>	
Negative	536 (27.1)
Positive	1444 (72.9)
Family relations	
Father	437 (22.1)
Mother	503 (25.4)
Brother	281 (14.2)
Sister	184 (9.3)
Paternal uncle	244 (12.3)
Paternal aunt	195 (9.8)
Maternal uncle	325 (16.4)
Maternal aunt	293 (14.8)
Paternal grand father	235 (11.9)
Paternal grand mother	221 (11.2)
Maternal grand father	264 (13.3)
Maternal grand mother	272 (13.7)

<sup>1</sup>Up to the third generation.

family history of DM. No significant difference was found in the metabolic characteristics of diabetic patients according to the family history of DM except for the SBP ( $P = 0.033$ ) and DBP ( $P = 0.025$ ). The development of T2DM complications was higher in patients with either a paternal or maternal history of DM than in those without; significantly higher for sleep loss (13.9% *vs* 6.7%, 12.2% *vs* 6.7%;  $P < 0.001$ ), hypertension (29.6% *vs* 20.9%, 22% *vs* 20.9%;  $P = 0.001$ ), retinopathy (17.6% *vs* 11.4%, 13.3% *vs* 11.4%;  $P = 0.006$ ) and antipathy (8% *vs* 4.7%, 7.7% *vs* 4.7%;  $P = 0.047$ ).

Table 4 shows physical and clinical characteristics and complications of T2DM according to family history of DM, while controlling for consanguinity. Out of 718, 556 (77.4%) of the diabetic patients of consanguineous parents had either a paternal (233/718; 32.5%) or maternal history (323/718; 45%) of DM; whereas family history of DM was lower in patients of non-consanguineous parents (888/1262; 70.4%). No significant difference was found in metabolic characteristics of patients according to the presence of DM in parents and relatives except for the SBP and DBP, found to be significantly higher among patients with maternal history of DM in the consanguineous group ( $P = 0.018$ ,  $P = 0.007$ , respectively). In addition, hypertension, retinopathy and antipathy were significantly higher among patients with a paternal history of DM in the consanguineous group ( $P = 0.002$ ,  $P = 0.007$ ,  $P = 0.003$ , respectively). No significant difference was found in T2DM complications of patients according to paternal/paternal history of DM in the non-consanguineous group.

Figure 1 shows the association between consanguinity and family history of DM in an Arab diabetic population in Qatar. The Venn diagram clearly shows the overlapping of parental consanguinity with a paternal and maternal history of diabetes mellitus.



**Figure 1** Association between consanguinity and family history of diabetes mellitus in an Arab diabetic population in Qatar ( $n = 1980$ ). DM: Diabetes mellitus.

## DISCUSSION

In the State of Qatar, as a result of changing lifestyle due to rapid urbanization, the prevalence of T2DM is increasing, as is observed worldwide. However, the role of genetic and environmental factors remains unclear. This is the first study to provide insight in the familial aggregation and transmission patterns of T2DM among an Arab population residing in Qatar. The study sample revealed that 72.9% of the subjects with DM had a positive family history of diabetes among at least one of their parents, siblings, uncles, aunts and grandparents. The degree of familial aggregation of diabetes among Tunisians<sup>[17]</sup> found that 70% of the diabetic patients had a positive family history of diabetes among at least one of their relatives from both sides, which is nearly identical to our study. A lower rate was observed in a French study<sup>[3]</sup> in which 66% of the diabetic patients had at least one relative with diabetes among their first and second degree relatives. Similar higher frequencies have also been reported among South Indians<sup>[18]</sup> (53.9%) and Pakistanis (70%)<sup>[19]</sup>. On the other hand, lower frequencies of positive family history have been reported by other studies in Asians<sup>[4]</sup> (36%), Europeans<sup>[20]</sup> (33%) and black South Africans (27%)<sup>[3]</sup>. In the study sample of 1980 diabetic patients, 71% reported at least one first degree familial member, which is similar to the study results of Crispim *et al*<sup>[21]</sup> (76.6%). These results support the strong familial aggregation of diabetes among an Arab population with a high prevalence among 1<sup>st</sup> degree relatives. Also, these study findings have proven that people with a family history of diabetes consider themselves to be at greater risk of developing diabetes in their offspring. These results are in agreement with a study by Hariri *et al*<sup>[22]</sup> that a family history of diabetes in a first-degree relative doubles a person's risk of developing diabetes.

Another important study finding was that the investigation of parental transmission patterns of T2DM showed an excess of maternal transmission of T2DM as mothers were implicated more frequently than fathers<sup>[23]</sup>. In the study sample, 25.4% of the mothers of the diabetic patients were diabetic compared to 22.1% of the fathers.

**Table 3** Physical, metabolic characteristics and complications among diabetic patients according to family history of diabetes mellitus (*n* = 1980)

Parameters	Family history <sup>1</sup>		No family history of DM ( <i>n</i> = 536)	<i>P</i> value
	Paternal history ( <i>n</i> = 635)	Maternal history ( <i>n</i> = 809)		
Age (yr)	44.9 ± 14	45.1 ± 15	46.7 ± 13.3	0.073
Duration of diagnosis (yr)	6.9 ± 4.2	7.3 ± 4.3	6.4 ± 3.3	0.001
BMI (kg/m <sup>2</sup> )	27.4 ± 4.9	26.8 ± 4.8	27.7 ± 4.9	0.033
Metabolic characteristics				
Systolic blood pressure (mmHg)	129.9 ± 19.5	129.1 ± 18.1	127.1 ± 14.7	0.033
Diastolic blood pressure (mmHg)	81.3 ± 11	80.4 ± 10.5	79.4 ± 8.5	0.025
Fasting glucose (mmol/L)	9.9 ± 8.0	9.1 ± 5.0	9.1 ± 4.7	0.092
HbA1c	8.2 ± 2.2	7.9 ± 2.1	8.1 ± 2.2	0.188
Serum urea level	5.9 ± 1.5	6.3 ± 2.3	5.8 ± 1.7	0.135
Serum creatinine (mmol/L)	77.2 ± 9.5	78.1 ± 9.8	73.8 ± 8.6	0.371
Total cholesterol (mmol/L)	5.0 ± 1.1	4.8 ± 1.1	4.9 ± 1.2	0.095
Serum alkaline phosphate	94.6 ± 11.3	101.7 ± 13.8	100 ± 12.4	0.402
T2DM complications, <i>n</i> (%)				
Sleep loss	88 (13.9)	99 (12.2)	36 (6.7)	< 0.001
Hypertension	167 (29.6)	156 (22.0)	111 (20.9)	0.001
Neuropathy	60 (9.4)	82 (10.1)	44 (8.2)	0.494
Retinopathy	112 (17.6)	108 (13.3)	61 (11.4)	0.006
Nephropathy	93 (14.6)	105 (13.0)	58 (10.8)	0.151
Antipathy	51 (8.0)	62 (7.7)	25 (4.7)	0.047

Data are expressed as absolute *n* (%) or mean ± SD. <sup>1</sup>Up to the third generation, two sided *P* values based on one way analysis of variance with *post hoc* Tukey's test for quantitative variables and  $\chi^2$  test for categorical variables. DM: Diabetes mellitus; T2DM: Type 2 diabetes mellitus; BMI: Body mass index; HbA1c: Hemoglobin A1c.

**Table 4** Physical and clinical characteristics of patients among consanguine and non consanguine parents (*n* = 1980)

Parameters	Consanguineous ( <i>n</i> = 718)				Non-consanguineous ( <i>n</i> = 1262)			
	Paternal history of DM	Maternal history of DM	Without familial history	<i>P</i> value	Paternal history of DM	Maternal history of DM	Without familial history	<i>P</i> value
	( <i>n</i> = 233)	( <i>n</i> = 323)	( <i>n</i> = 162)		( <i>n</i> = 402)	( <i>n</i> = 486)	( <i>n</i> = 374)	
Age (yr)	43.9 ± 14.7	45.1 ± 15.4	47.3 ± 13.4	0.058	45.5 ± 13.6	45.1 ± 14.8	46.3 ± 13.3	0.532
BMI (kg/m <sup>2</sup> )	27.4 ± 4.8	26.6 ± 4.9	28.1 ± 4.9	0.028	27.4 ± 5.0	26.9 ± 4.8	27.6 ± 4.8	0.428
Duration of DM (yr)	6.6 ± 3.9	7.2 ± 4.4	5.9 ± 2.9	0.004	7.2 ± 4.3	7.4 ± 4.2	6.7 ± 3.5	0.065
Metabolic characteristics								
Systolic BP (mmHg)	129.6 ± 20.4	130.7 ± 17.9	125.9 ± 11.2	0.018	130.1 ± 19.1	128.2 ± 18.1	127.8 ± 16.5	0.224
Diastolic BP (mmHg)	81.3 ± 11.4	81.1 ± 10.7	78.2 ± 8.0	0.007	81.2 ± 11.4	79.9 ± 10.4	80.0 ± 9.1	0.220
Fasting glucose (mmol/L)	10.3 ± 8.9	8.9 ± 5.0	8.9 ± 5.4	0.071	9.7 ± 9.6	9.3 ± 5.0	9.3 ± 4.3	0.689
HbA1c (%)	7.8 ± 2.1	7.7 ± 2.1	7.8 ± 2.3	0.900	8.3 ± 2.4	7.9 ± 2.2	8.3 ± 2.1	0.121
Serum urea level	5.8 ± 3.0	6.2 ± 3.2	5.9 ± 4.4	0.622	5.9 ± 2.7	6.3 ± 3.1	5.7 ± 2.8	0.170
Creatinine (mmol/L)	71.9 ± 8.5	78.0 ± 9.8	72.4 ± 9.4	0.313	75.5 ± 9.9	76.2 ± 10.2	70.9 ± 8.6	0.372
Tot. cholesterol (mmol/L)	4.9 ± 1.1	4.8 ± 1.1	4.9 ± 1.2	0.626	5.0 ± 1.2	4.8 ± 1.1	4.9 ± 1.3	0.173
Serum alkaline phosphate	92.2 ± 11.1	99.3 ± 12.8	93.5 ± 12.2	0.683	95.9 ± 12.5	102.9 ± 13.2	104.6 ± 13.5	0.460
T2DM complications, <i>n</i> (%)								
Sleep loss	44 (18.9)	40 (12.4)	12 (7.4)	0.003	44 (10.9)	59 (12.1)	24 (6.4)	0.017
Hypertension	67 (32.5)	59 (20.6)	31 (19.1)	0.002	100 (27.9)	97 (22.9)	80 (21.7)	0.114
Neuropathy	19 (8.2)	34 (10.5)	12 (7.4)	0.446	41 (10.2)	48 (9.9)	32 (8.6)	0.712
Retinopathy	44 (18.9)	41 (12.7)	13 (8.0)	0.007	68 (16.9)	67 (13.8)	48 (12.8)	0.231
Nephropathy	29 (12.4)	40 (12.4)	13 (8.0)	0.303	64 (15.9)	65 (13.4)	45 (12)	0.276
Antipathy	21 (9.0)	21 (6.5)	4 (2.5)	0.003	30 (7.5)	41 (8.4)	21 (5.6)	0.284
Hypoglycemia	62 (26.6)	91 (28.2)	46 (28.4)	0.899	111 (27.6)	113 (23.3)	99 (26.5)	0.299
Impotence	15 (6.4)	17 (5.3)	3 (1.9)	0.104	33 (8.2)	38 (7.8)	19 (5.1)	0.180

Data are expressed as absolute *n* (%) or mean ± SD. Two sided *P* values based on one way analysis of variance with *post hoc* Tukey's test for quantitative variables and  $\chi^2$  test for categorical variables. DM: Diabetes mellitus; T2DM: Type 2 diabetes mellitus; BMI: Body mass index; HbA1c: Hemoglobin A1c; BP: Blood pressure.

Consistent with our results, a higher frequency of positive family history among mothers than fathers was reported in studies conducted in Brazil<sup>[21]</sup> (48.4% *vs* 21.3%), Britain<sup>[23]</sup> (36% *vs* 15%), France<sup>[24]</sup> (33% *vs* 17%), Greece<sup>[25]</sup>

(27.7% *vs* 11%) and Tunisia<sup>[17]</sup> (21% *vs* 10%).

The present study extends the scope of genetic influence on DM by including parents, siblings, uncles, aunts and grandparents in the familial history. It was observed

that 83.7% of the diabetic patients have an affected mother and at least one relative on the maternal side, compared to only 67.3% of diabetic patients with an affected father and family member on the paternal side, suggesting a maternal transmission of T2DM in the Arab population. The excess maternal transmission of T2DM reported in this study is in line with studies from different populations with varying frequencies<sup>[3,4,18,21,25]</sup>. A positive family history of T2DM was more common among maternal aunts/uncles (31.2%) than in paternal aunts/uncles (22.2%), showing that this maternal effect likely extends to the previous generation in 2<sup>nd</sup> degree relatives, as reported in another study<sup>[21]</sup>. These study results support the existence of excess of maternal transmission of T2DM in their population. On the contrary, in the Framingham population study<sup>[26]</sup>, maternal and paternal diabetes conferred equivalent risk for occurrence of T2DM in offspring. In contrast to these findings, McCarthy *et al.*<sup>[27]</sup> found no difference in parental transmission of T2DM in a population with high prevalence of diabetes. Longer average life span in women could increase the likelihood that mothers develop T2DM. Fathers may have more undetected diabetes because of reduced screening rates and health care utilization or may develop diabetes at an older age than mothers.

In our study, there was an early onset of diabetes among patients with a family history of diabetes in the age group 18-30 years (24%) compared to other patients whose parents were non-diabetic (13.4%), which is similar to the results found in Greek diabetic patients<sup>[25]</sup>; this study reported that the presence of a family history of diabetes results in an early onset of the disease in the offspring. Younger age of the onset of diabetes had been noted, which implies that these subjects develop diabetes in the most productive years of their life and have a greater chances of developing complications<sup>[28]</sup>. Crispim *et al.*<sup>[21]</sup> reported in their study that when the disease is diagnosed at an early age, the genetic component is more important to its development.

In the study sample, a positive family history of DM was more common among diabetic patients of consanguineous parents (77.4%) with high prevalence of maternal history (45%), whereas it was lower in patients of non-consanguineous parents (70.4%). It was reported in a recent study by Bener *et al.*<sup>[10]</sup> that there was a significant increase in the prevalence of diabetes mellitus in consanguineous couples in Qatar. The current data showed that consanguinity increased the family history of DM in patients. This means that consanguinity is an important factor in the causation of diabetes mellitus in offspring.

The influence of various transmission patterns of T2DM on metabolic factors and diabetic complications have been examined. Results showed no significant difference in clinical parameters between patients with a parental or maternal history of diabetes in the study sample except for hypertension, which is similar to the study results by Bo *et al.*<sup>[29]</sup>. A study in Tunisia<sup>[17]</sup> showed no significant difference in clinical parameters between patients with paternal or maternal history of diabetes in the studied sample. The development of sleep loss, hypertension,

retinopathy and antipathy were significantly higher in the studied patients with a family history of DM than those without. Jali *et al.*<sup>[28]</sup> found retinopathy and neuropathy less in patients with a family history of DM and risk was same in both the groups with respect to nephropathy.

Harrison *et al.*<sup>[5]</sup> documented that family history information may serve as a useful tool for public health because it reflects both genetic and environmental factors. Examining family history of DM may be a valuable approach for identifying patients at risk for diabetes. In addition, this survey provides some indication that knowledge of family history of diabetes may lead to identifying people at increased risk of diabetes and perhaps motivate them to make preventive life style changes that could favorably affect both clinical practice and patient behavior.

In conclusion, the study findings showed an excess of maternal transmission of T2DM in a sample of an Arab diabetic population residing in Qatar. The data support the dominant maternal role in the development of diabetes mellitus in their offspring. No significant difference was observed between maternal and paternal diabetes in metabolic characteristics except for hypertension. Complications were higher in diabetic patients with a family history of DM. Family history of DM was higher in patients of consanguineous parents compared to non-consanguineous parents. The presence of a family history of diabetes resulted in an early onset of the disease of the offspring. Interventions to change life style habits among families might reduce the risk of diabetes in the offspring of diabetic patients.

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## COMMENTS

### Background

Diabetes is a disease that has a strong clustering in families and has a genetic component. Family history is a well-known risk factor for developing type 2 diabetes mellitus (T2DM). The high incidence of consanguineous marriages in the State of Qatar highlighted the importance of determining the influence of familial history of T2DM in the offspring.

### Research frontiers

The study indicated that knowledge of family history of diabetes may lead to identifying people at increased risk of diabetes. The study highlighted the importance of identifying this high risk group and make preventive life style changes which might reduce the risk of diabetes in offspring.

### Innovations and breakthroughs

The important study findings of this article are compared to studies conducted regionally and internationally which make the readers understand the high prevalence of diabetes mellitus in a consanguineous population.

### Applications

This will encourage the researchers in this region to explore the paternal transmission of T2DM in their community and conduct intervention studies to change life style habits among families.

### Peer review

The authors recommended through this study that family history information may serve as a useful tool for public health because it reflects both genetic and

environmental factors. Physicians should consider the family history of diabetes mellitus to identify the onset of DM in their offspring.

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## Contents

Bimonthly Volume 4 Number 3 June 15, 2013

<b>FIELD OF VISION</b>	47	Bariatric surgery-mediated weight loss and its metabolic consequences for type-2 diabetes <i>Raghow R</i>
<b>REVIEW</b>	51	Gastrointestinal complications of diabetes mellitus <i>Krishnan B, Babu S, Walker J, Walker AB, Pappachan JM</i>
<b>BRIEF ARTICLE</b>	64	Cardiometabolic health among gastric bypass surgery patients with polycystic ovarian syndrome <i>Gomez-Meade CA, Lopez-Mitnik G, Messiah SE, Arheart KL, Carrillo A, de la Cruz-Muñoz N</i>
	70	Caribbean maitotoxin elevates $[Ca^{2+}]_i$ and activates non-selective cation channels in HIT-T15 cells <i>Lu XZ, Deckey R, Jiao GL, Ren HF, Li M</i>
	76	Hemoglobin A1c in early postpartum screening of women with gestational diabetes <i>Katreddy MV, Pappachan JM, Taylor SE, Nevill AM, Indusekhar R, Nayak AU</i>
	82	Atherosclerotic cardiovascular disease among patients with type 2 diabetes in Basrah <i>Mansour AA, Ajeel NAH</i>

**APPENDIX** I-V Instructions to authors

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## Bariatric surgery-mediated weight loss and its metabolic consequences for type-2 diabetes

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### Abstract

The worldwide epidemic of obesity and its medical complications are being dealt with a combination of life style changes (*e.g.*, healthier diet and exercise), medications and a variety of surgical interventions. The Roux-en Y gastric bypass (RYGB) and laparoscopic adjustable gastric banding (LAGB) are two of the most common weight loss surgeries for morbid obesity-associated metabolic syndrome and insulin resistance. A vast majority of patients that undergo RYGB and LAGB are known to experience marked weight loss and attenuation of diabetes. A number of recent studies have indicated that the rates of remission in glycemic control and insulin sensitivity are significantly greater in patients that have undergone RYGB. A plausible hypothesis to explain this observation is that the gastric bypass surgery as opposed to the gastric banding procedure impinges on glucose homeostasis by a weight loss-independent mechanism. In a recent paper, Bradley *et al* have experimentally explored this hypothesis. The authors compared several clinical and laboratory parameters of insulin sensitivity and  $\beta$ -cell function in cohorts of RYGB and LAGB patients before and after they lost approximately 20% of their body mass. After

weight loss, both groups of patients underwent similar changes in their intra-abdominal and total adipose tissue volume, hepatic triglyceride and circulating leptin levels. The RYGB patients who lost 20% body mass, manifested higher postprandial output of glucose, insulin and glucagon-like peptide-1; these laboratory parameters remained unchanged in LAGB patients. Irrespective of the observed differences in transient responses of RYGB and LAGB patients to mixed meal, the overall glycemic control as judged by glucose tolerance, multi-organ insulin sensitivity and  $\beta$ -cell function were nearly identical in the two groups. Both RYGB and LAGB patient cohorts also experienced similar changes in the expression of a number of pro- and anti-inflammatory markers. Based on these analyses, Bradley *et al* concluded that similar restoration of insulin sensitivity and  $\beta$ -cell function in non-diabetic obese patients that have undergone RYGB and LAGB were directly due to marked weight loss. These data have important implications for the risk/benefit analysis of weight loss therapy by bariatric procedures.

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**Key words:** Bariatric surgery; Roux-en Y gastric bypass; Laparoscopic adjustable gastric banding; Weight loss; Type-2 diabetes

**Core tip:** This report demonstrates that the positive effects of Roux-en Y gastric bypass and laparoscopic adjustable gastric banding are mainly caused by weight loss. Quantitatively similar losses of intra-abdominal and total adipose were seen in both groups of patients who also experienced improved glucose tolerance, multi-organ insulin sensitivity and cell function. Weight loss was associated with positive changes in a number of pro- and anti-inflammatory markers, regardless of the type of gastric surgery. In light of these findings the risk/benefit ratio of weight loss therapy by bariatric procedures with varying degrees of invasiveness, post-surgical complications and cost need to be re-evaluated.

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## COMMENTARY ON HOT TOPICS

Obesity-associated type-2 diabetes mellitus (T2DM) and metabolic syndrome, and the cardiovascular consequences of chronic obesity are steadily emerging as key global healthcare challenges of the 21<sup>st</sup> century (World Health Organization Global Infobase: data on overweight and obesity mean body mass index, healthy diets and physical inactivity; [www.who.int/mediacentre/](http://www.who.int/mediacentre/)). Surgical procedures such as gastric banding, gastric bypass and biliopancreatic diversion/duodenal switch have proven to be highly effective therapies for weight loss in morbidly obese individuals<sup>[1-4]</sup>. Regardless of whether weight loss is achieved by a combination of diet and/or exercise, or by surgery, such interventions, invariably, lead to improved metabolic profiles and amelioration of diabetes.

A comprehensive review and meta-analysis of 621 studies involving different types of bariatric surgeries revealed that a vast majority of patients that underwent weight loss following these procedures also experienced improvement in the clinical and laboratory manifestations of their diabetes<sup>[5]</sup>. Furthermore, it was noted that the improved glucose homeostasis and weight loss were progressively more significant with laparoscopic adjustable gastric banding (LAGB), gastropasty, Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion/duodenal switch procedures<sup>[5]</sup>. Whether variable metabolic outcome of different types of bariatric procedures is caused by weight loss alone or involves other factors remains controversial. Two common bariatric surgeries used for weight loss therapy are RYGB that diverts the ingested food from passage through the upper gastrointestinal (GI) tract<sup>[6]</sup> and the laparoscopic adjustable gastric banding (LAPG) technique that reduces the size of the stomach<sup>[7]</sup>. The meta-analytical observations of Buchwald *et al*<sup>[5]</sup> and a number of other experimental findings have led some investigators to question the exclusive cause and effect relationship between weight loss and diabetes in patients undergoing bariatric surgeries<sup>[8,9]</sup>. In light of these data, it has been posited that bariatric interventions impinge on the mechanisms of glucose homeostasis that may be independent of weight loss<sup>[8,10]</sup>. However, unequivocal experimental data that support this tantalizing hypothesis are currently missing. In the December 2012 issue of the *Journal of Clinical Investigation*, Bradley *et al*<sup>[11]</sup> have described a set of experiments that were specifically aimed at testing this hypothesis. Based on these data authors concluded that upper GI tract diversion by RYGB improved insulin sensitivity and  $\beta$ -cell function by a weight loss-dependent mechanism.

Bradley *et al*<sup>[11]</sup> recruited two groups of obese subjects,

ten in each cohort, that were insulin-resistant, as judged by homeostasis model assessment of insulin resistance (HOMA-IR) values of  $> 2.5$ . The choice of insulin-resistant obese patients that were not diabetic was made specifically with a goal to minimize the confounding variables of baseline glycemic control, glucose toxicity and interference with medications used to treat diabetes. The patient cohorts underwent RYGB or LAGB surgeries and were allowed to reach the target weight loss of 20% at  $22 \pm 7$  and  $16 \pm 2$  wk, respectively. The clinical and laboratory measurements that included body composition, insulin sensitivity and metabolic response to mixed meal were carried out in both groups of patients, before and after weight loss. These analyses revealed that total fat mass, intra-abdominal adipose tissue volume, intrahepatic triglyceride content and plasma leptin concentration were altered similarly in RYGB and LAGB subjects after weight loss. Both cohorts of patients also elicited similar beneficial changes in the steady state levels of their plasma glucose, C-peptide, adiponectin and C-reactive protein.

Bradley *et al*<sup>[11]</sup> noted that following approximately 20% weight loss, the HOMA-IR scores decreased by more than 2-fold in both LAGB and RYGB patients who also displayed similar reductions in total insulin secretion rates (ISR) and total  $\beta$ -cell sensitivity. However, the kinetics of plasma glucose concentration after a mixed-meal was significantly different in RYGB and LAGB cohorts after target weight loss. Thus, postprandial rate of appearance (Ra) of glucose in RYGB patients increased from  $70\% \pm 19\%$  to  $92\% \pm 2\%$ , before and after weight loss, respectively; the RYGB patients also showed a higher peak in the rise of plasma insulin, C-peptide, and a marked increase in glucagon-like peptide-1 (GLP-1) after a mixed meal. The higher values of glucose in RYGB patients after weight loss likely reflected a more rapid emptying of their meal into small intestine; a higher dynamic ISR in these patients reflected a rapid rise in circulating glucose combined with increased plasma GLP-1. This explanation of greater dynamic ISR in RYGB patients is reasonable since postprandial rates of endogenous glucose production (EGP) were similar before and after weight loss in LAGB patients. In contrast, following a mixed meal, RYGB subjects elicited a faster and almost complete suppression of EGP that also rapidly returned to baseline. These differences in kinetics of EGP underscore a critical role of hepatic gluconeogenesis in preventing postprandial hypoglycemia in RYGB patients. The observed differences Ra to mixed meal notwithstanding, the area under the curve measurements of plasma insulin and C-peptide values, decreased to a similar extent in LAGB and RYGB patients after undergoing weight loss; both groups of patients also exhibited a near doubling of their disposition index (DI). It should be noted that the plasma concentration of glucagon did not change in either group of patients.

The authors observed that the whole body rise in insulin sensitivity, as judged by a 25% decline in insulin secretion in response to oral glucose challenge, occurred in

both LAGB and RYGB patients. However, as assessed by DI, there was a 2-fold enhancement of insulin sensitivity. Thus,  $\beta$ -cell function, assessed as total meal-induced insulin secretion in relationship to DI increased by about 75% in both LAGB and RYGB patients. Based on these data strongly suggested that weight loss, regardless of whether it occurred as a result of upper GI tract diversion or gastric banding could restore  $\beta$ -cell function, insulin sensitivity and oral glucose tolerance in non-diabetic patients.

Since a 5%-10% weight loss was shown to be insufficient to alter insulin sensitivity of skeletal muscle in previous studies<sup>[8,12-14]</sup>, Bradley *et al*<sup>[11]</sup> speculated that for skeletal muscle to become more insulin sensitive a more marked weight loss is needed. The data in the current study support this notion as judged by nearly 2-fold improvement in skeletal muscle insulin sensitivity after 20% weight loss in both LAGB and RYGB patients. It should be pointed out however, that weight loss in either group of patients did not change the intramyocellular content of diacylglycerol or ceramide; these two lipids have been associated with skeletal muscle insulin resistance in rodents<sup>[15,16]</sup>.

Finally, Bradley *et al*<sup>[11]</sup> reported that weight loss following either RYGB or LAGB led to amelioration of pro-inflammatory factors putatively involved in aberrant regulation of metabolism in morbidly obese animals and man. They measured the steady state levels of mRNA encoding EMR1 and CD11B, cell surface markers of pro-inflammatory macrophages as well as the expression of pro-inflammatory cytokines [*e.g.*, colony-stimulating factor, interleukin-6 (IL-6), tumor necrosis factor- $\alpha$ , and leptin]. Weight loss led to reduced expression of markers of inflammation and a concomitant enhancement of expression of IL-10, an anti-inflammatory cytokine. It is noteworthy that concomitant up-regulation of pro-inflammatory and down-regulation of anti-inflammatory signals occurred to a similar extent in both RYGB and LAGB patients. These observations demonstrate that in addition to restoring insulin sensitivity and  $\beta$ -cell function, weight loss impinges on the pathways of inflammation known to exacerbate insulin resistance and T2DM.

In conclusion, the data of Bradley *et al*<sup>[11]</sup> indicate that marked weight loss in obese subjects is accompanied by changes in key parameters of postprandial glucose homeostasis, multi-organ insulin sensitivity,  $\beta$ -cell function and adipose tissue inflammation. The authors' assertion that manifestly different response of RYGB patients to a mixed meal, as judged by increased transient levels of plasma glucose, insulin and GLP-1 are unlikely to play a therapeutic role in alleviating insulin resistance is reasonable. Based on these data authors concluded that restoration of  $\beta$ -cell function and insulin sensitivity occurred primarily as a result of weight loss. Although this study employed non-diabetic obese patients who underwent RYGB- or LAGB-dependent weight loss, the insights of this excellent study are highly relevant to the causes and consequences of morbid obesity-associated T2DM and

its attenuation by weight loss. The data of this study also have serious implications for the choices of bariatric procedures that differ in their pre- and post-surgical preparations and complications and cost.

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## Gastrointestinal complications of diabetes mellitus

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### Abstract

Diabetes mellitus affects virtually every organ system in the body and the degree of organ involvement depends on the duration and severity of the disease, and other co-morbidities. Gastrointestinal (GI) involvement can present with esophageal dysmotility, gastro-esophageal reflux disease (GERD), gastroparesis, enteropathy, non alcoholic fatty liver disease (NAFLD) and glycogenic hepatopathy. Severity of GERD is inversely related to glycemic control and management is with prokinetics and proton pump inhibitors. Diabetic gastroparesis manifests as early satiety, bloating, vomiting, abdominal pain and erratic glycemic control. Gastric emptying scintigraphy is considered the gold standard test for diagnosis. Management includes dietary modifications, maintaining euglycemia, prokinetics, endoscopic and surgical treatments. Diabetic enteropathy is also common and management involves glycemic control and symptomatic measures. NAFLD is considered a hepatic manifestation of metabolic syndrome and treatment is

mainly lifestyle measures, with diabetes and dyslipidemia management when coexistent. Glycogenic hepatopathy is a manifestation of poorly controlled type 1 diabetes and is managed by prompt insulin treatment. Though GI complications of diabetes are relatively common, awareness about its manifestations and treatment options are low among physicians. Optimal management of GI complications is important for appropriate metabolic control of diabetes and improvement in quality of life of the patient. This review is an update on the GI complications of diabetes, their pathophysiology, diagnostic evaluation and management.

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**Key words:** Gastrointestinal complications; Diabetes mellitus; Esophageal complications; Nonalcoholic fatty liver disease; Diabetic gastroparesis; Diabetic enteropathy; Glycogenic hepatopathy

**Core tip:** Although relatively common, gastrointestinal (GI) complications of diabetes mellitus are under-recognized by most physicians. Early identification and prompt management of GI complications are of paramount importance as they are associated with significant morbidity. Common GI complications are esophageal dysmotility, gastro-esophageal reflux disease, gastroparesis, enteropathy, non alcoholic fatty liver disease (NAFLD) and glycogenic hepatopathy. Damage to the myenteric neurons due to longstanding diabetes causes esophageal, gastric and enteric disease. NAFLD is a hepatic manifestation of metabolic syndrome and is commonly seen in type 2 diabetes while glycogenic hepatopathy is due to poor glycemic control in type 1 diabetes. Clinical manifestations, pathogenesis, diagnostic evaluation and management of GI complications of diabetes are discussed in this article.

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## INTRODUCTION

The prevalence of diabetes mellitus has now reached epidemic proportions in both developed and developing countries, affecting more than 366 million people worldwide<sup>[1]</sup>. This number is likely to increase in the coming years as a result of an ageing global population, urbanization, rising prevalence of obesity and sedentary lifestyles. Diabetes affects virtually every organ system in the body and the duration and severity of the disease may have a direct impact on organ involvement. Though gastrointestinal (GI) complications are common in longstanding diabetes, the awareness of these complications is low among physicians. Early identification and appropriate management of GI complications are important for improving both diabetic care and quality of life of the affected patient. This review aims to outline the GI complications of diabetes and the latest management options.

## ESOPHAGEAL COMPLICATIONS

The thoracic esophagus and lower esophageal sphincter (LES) are composed of smooth muscle fibres innervated by myenteric plexus, and these autonomic nerves can be affected by diabetic neuropathy in patients with longstanding diabetes. Autonomic neuropathy and structural remodeling of the esophageal musculature in diabetes results in abnormal peristalsis, spontaneous contractions and reduced LES tone<sup>[2]</sup>. Morphological and biomechanical properties of the esophagus have been found to be altered significantly in animal models of diabetes<sup>[3]</sup>. The prevalence of esophageal dysmotility in diabetes has been reported to be as high as 63%<sup>[4]</sup>. The same study also found that there was no difference in dysmotility between patients with type 1 and type 2 diabetes or between genders and there was a strong association with retinopathy. Patients with dysmotility had longer duration of diabetes compared with those without dysmotility. Although the prevalence of esophageal dysmotility is high among patients with diabetes, only a minority present with the classical symptoms of dysphagia and heartburn<sup>[5]</sup>.

The prevalence of gastroesophageal reflux symptoms in diabetes could be as high as 41%<sup>[6]</sup>. Erosive esophagitis (EE) was more frequent (66.7%) in diabetic patients with neuropathy than those without neuropathy (33.3%); also asymptomatic EE was significantly more frequent in the same group. In patients with type 2 diabetes, peripheral neuropathy is an independent risk factor for EE; however patients may be asymptomatic and a gastroscopy may be recommended in these patients<sup>[7]</sup>. Circulating levels of adiponectin, a potential anti-inflammatory adipocytokine is inversely related to visceral fat accumulation and it has been shown that the prevalence of gastroesophageal reflux disease is higher in type 2 diabetic patients with metabolic syndrome and low levels of serum adiponectin<sup>[8]</sup>.

Diagnosis of reflux and dysmotility has relied on esophageal pH monitoring and conventional manometry for many years. The use of the wireless Bravo pH capsule, which allows catheter-free monitoring and imped-

ance-pH measurement, a catheter-based technique which allows detection of acid and non-acid reflux have been major developments in the diagnostic field recently<sup>[9]</sup>. Two new procedures are available to assess esophageal motility: high resolution manometry which uses many pressure sensors and provides spatiotemporal plots of esophageal pressure changes; and impedance manometry, a test that directly measures bolus transit and provides conventional manometric data<sup>[9]</sup>.

Gastroesophageal reflux disease was found to be inversely related to glycemic control and better glycemic control may improve esophageal dysmotility and reflux<sup>[10]</sup>. Management of reflux disease involves prokinetic drugs, such as metoclopramide and proton pump inhibitors. A two-week course of erythromycin has been shown to reduce mean esophageal transit time and gastric emptying time in type 2 diabetics<sup>[11]</sup>. Patients are also advised to drink fluids immediately after taking medications to avoid pill-induced esophagitis.

## GASTROPARESIS

Gastroparesis, one of the commonest GI complications of diabetes mellitus, produces symptoms of gastric retention in the absence of physical obstruction<sup>[12]</sup>. The incidence of gastroparesis in a population with diabetes is reportedly low (5.2% over 10 years in type 1 and 1% in type 2 diabetes), but greater than in the general population (0.2%)<sup>[13]</sup>. Delayed gastric emptying can be demonstrated in 27%-65% of patients with type 1 diabetes and about 30% of patients with type 2 diabetes<sup>[14]</sup>. The incidence of gastroparesis is higher in women<sup>[15]</sup>. A recent study has reported obesity as a significant independent predictor of symptoms suggestive of gastroparesis in patients with type 2 diabetes mellitus (T2DM) and neuropathy<sup>[16]</sup>.

### Pathogenesis

The pathogenesis of diabetic gastroparesis is multifactorial and currently poorly understood. Delayed gastric emptying may be the first indication of gastroparesis in diabetes<sup>[15]</sup>. Elevated glycated hemoglobin level, duration of diabetes in excess of 10 years and the presence of macro- and microvascular complications are all accepted risk factors for the development of diabetic gastroparesis. Delayed gastric emptying contributes to poor glycemic control and may be the first indication that the patient is developing gastroparesis. Loss of the normal Migrating Motor Complexes, blunted antral contractions, spasm of the pylorus and small intestine and poor meal accommodation in the stomach are all demonstrable in diabetes<sup>[12]</sup>. Other factors that may have a role in the pathogenesis includes impaired inhibitory nitric oxide containing nerves, absent or dysmorphic interstitial cells of Cajal, smooth muscle fibrosis and abnormal macrophage-containing immune infiltrates<sup>[17,18]</sup>. Bezoar formation can contribute to the development of gastroparesis in some individuals. Endoscopic biopsies from diabetic gastroparesis demonstrate abnormal mucosal nerve density and morphology, reflecting possible potential for endoscopic diagnosis

of enteric neuropathy<sup>[19]</sup>. Neurohumoral factors including glucagon-like peptide-1 (GLP-1) can play a role in gastroparesis and the use of GLP-1 agonists Exenatide and Liraglutide can lead to symptoms of gastroparesis. A recent study showed that deficiency of apolipoprotein E can be a risk factor in diabetic gastroparesis in an animal model<sup>[20]</sup>. Extrinsic factors such as medications as well as concomitant disorders such as anxiety and depression may result in increased reporting of symptoms.

### Clinical features

Symptoms of gastroparesis include nausea, vomiting, early satiety, postprandial fullness, bloating and upper abdominal pain. Worsening glycemic control along with frequent hypoglycemic episodes or unexplained alternating hyper- and hypoglycemia due to a mismatch between insulin action and carbohydrate absorption should prompt the clinician to evaluate the patient for diabetic gastroparesis. About 53% of patients may experience weight loss but 18%-24% may experience weight gain<sup>[14]</sup>. More than half of affected individuals present with acute onset of symptoms and the others insidiously. One third of cases have chronic symptoms with periodic exacerbations and one third have chronic worsening symptoms<sup>[14]</sup>. Epigastric distention and succussion splash may be observed in some patients but physical examination may not be always helpful.

### Evaluation

A technical review from the American Gastroenterological Association recommends performing an initial evaluation consisting of careful history taking and physical examination, followed by complete blood count, thyroid stimulating hormone test, metabolic panel and optional amylase and pregnancy test<sup>[21]</sup>. History taking should particularly focus on macro- and micro-vascular complications of diabetes, although gastroparesis may occur in their absence. Additionally rumination syndrome should be excluded. Physical examination should focus on looking for evidence of peripheral and autonomic neuropathy, epigastric distension and the presence of succussion splash one hour post mealtimes. This is followed by upper GI endoscopy to rule out mechanical obstruction. Alternatively, an upper GI series with small bowel follow-through or small bowel magnetic resonance imaging can be performed. In the presence of significant abdominal pain, an abdominal ultrasound scan should be carried out to rule out biliary colic<sup>[16]</sup>. Presence of food in the stomach at endoscopy following a 12-h fast, in the absence of gastric outlet obstruction, is strongly suggestive of gastroparesis.

The diagnosis of gastroparesis is made by gastric emptying scintigraphy using <sup>99m</sup>Tc sulphur colloid bound to solid food<sup>[21]</sup>. This noninvasive, quantitative method is considered the gold standard test for diagnosing gastroparesis. The patient ingests a technetium-labeled egg meal and gastric emptying is then measured by scintiscanning at 15-min intervals for 4 h. However this test lacks standard-

ization. A newer four image simplified scanning method has also shown comparable results<sup>[22]</sup>. The American Neurogastroenterology and Motility Society recommended a test meal of two slices of bread with jam plus two large eggs labeled with technetium-99m sulphur colloid and scintigraphy carried out at 0, 1, 2 and 4 h post prandially. A diagnosis of gastroparesis can be made if there is > 90% retention at 1 h, > 60% at 2 h and > 10% at 4 h<sup>[23]</sup>.

An alternative method for gastric emptying study uses an indigestible wireless motility capsule (WMC), which senses intraluminal pH, temperatures and pressures as it traverses the gastrointestinal tract. The capsule wirelessly transmits the data to a receiver worn by the patient until it is excreted. WMC gastric emptying times greater than 5 h are said to be delayed, and this correlates with scintigraphic measurements<sup>[24]</sup>. Non-radioactive <sup>13</sup>C-breath tests quantify exhaled <sup>13</sup>CO<sub>2</sub> after duodenal assimilation of a standardized substrate (octanoate, spirulina platensis) and are an alternative to scintigraphy<sup>[25]</sup>. The main advantage of these newer technologies is the lack of radiation exposure; however their general availability is limited.

Selected patients can be offered additional testing to exclude other contributions to the symptoms. Antroduodenal manometry excludes small bowel dysmotility, found in 17%-85% of gastroparetics<sup>[12]</sup>. Electrogastrography (EGG) can be used to detect rhythm disruptions and blunted postprandial responses. However a recent study has demonstrated the relative insensitivity of clinical EGG methodologies<sup>[26]</sup>.

### Treatment

Gastroparesis treatments include general measures, dietary modifications, medications that enhance emptying or lessen vomiting, non-medication interventions, psychological therapies and consideration of more invasive surgical treatment<sup>[12]</sup>. A grading system for assessing severity and guiding the management of gastroparesis has been suggested (Table 1)<sup>[27]</sup>.

### General approaches and dietary modifications

General approaches to management of gastroparesis include ensuring good hydration, correcting electrolyte imbalances, management of glycemic control and symptom reduction with pharmacotherapeutic agents. Any medications that can delay gastric emptying should be discontinued if possible. Dietary modifications include increasing liquid-based meals (as the rate of emptying liquid from the stomach is usually the same in diabetic gastroparesis), reducing fat and non-digestible fibre intake, avoiding large meals with high calorie contents and ensuring small frequent meals spread throughout the day.

Maintaining euglycemia has been one of the main principles of managing diabetic gastroparesis. Prolonged postprandial hyperglycemia has been observed in patients with diabetic gastroparesis compared to those with normal gastric emptying<sup>[28]</sup>. Another study observed a reduction of 1.8% in hemoglobin A<sub>1c</sub> after initiating insulin

**Table 1 Classification of severity of gastroparesis**

Grade 1: Mild	Symptoms easily controlled Regular diet/minor dietary modifications helps to maintain normal nutritional status
Grade 2: Compensated	Moderate symptoms that are reasonably controlled with prokinetics and anti-emetics Maintenance of nutrition with diet/lifestyle changes Hospitalizations-infrequent
Grade 3: Gastric failure	Refractory symptoms Inadequate nutrition Needing hospitalization for therapy and nutritional supplementation (either enteral or parenteral) May need surgical or endoscopic intervention or gastric "pacemaker"

Originated from Abell *et al*<sup>[27]</sup>.

**Table 2 Drugs useful in treatment of diabetic gastroparesis**

Drug/drug group	Mechanism of action	Common side effects	Efficacy
Metoclopramide 10 mg 4 times/d	Anti-emetic, reduces nausea and post-prandial fullness, increases gastro-esophageal sphincter tone and improves antro-pyloro-duodenal coordination	Tardive dyskinesia, drowsiness, irritability, extrapyramidal symptoms and dystonic reactions	Symptom control in 1/3 to 2/3 of patients
Domperidone 10 -20 mg 3 times/d	Similar to metoclopramide with fewer CNS side effects due to a predominant peripheral mechanism of action	May prolong QTc interval in ECG; in turn may provoke cardiac arrhythmia	Effective in up to 60% of cases; tachyphylaxis develops in a few weeks requiring discontinuation
Erythromycin 50-250 mg thrice daily	Motilin receptor agonist. Reduces gastric emptying time	Nausea and vomiting at high doses	Modest symptom control Intravenous form can be useful in refractory vomiting
Promethazine, prochlorperazine and chlorpromazine	Mechanism of antiemesis poorly understood	Drowsiness, liver injury and extrapyramidal effects	Marginal improvement of symptoms Intramuscular chlorpromazine is very effective in refractory vomiting
Ondansetron	Central serotonin receptor (5-HT <sub>3</sub> ) antagonist Inhibits vagus nerve	Extrapyramidal effect	Modest efficacy

Originated from Hasler<sup>[12]</sup>. CNS: Central nervous system; ECG: Electrocardiogram; QTc: Corrected QT interval.

pump therapy<sup>[29]</sup>. This eventually reduced the number and length of hospitalizations for diabetic gastroparetics.

**Prokinetics**

Prokinetics are medications that augment gastrointestinal motility. In general these increase gastric motility and enhance stomach emptying. Medications commonly used in treatment are shown in Table 2.

Mosapride is a selective 5-HT<sub>4</sub> agonist that accelerates gastric emptying. Orally administered mosapride citrate has been associated with significantly increased food intake in ob/ob obese mice, with a tendency to decrease fasting blood glucose and fructosamine concentrations compared with controls<sup>[30]</sup>. A recent study reported symptom reductions in interferon induced gastroparesis in hepatitis C patients, treated with mosapride<sup>[31]</sup>. Other agents with gastric stimulating effects in gastroparesis include the new 5-HT<sub>4</sub> agonists prucalopride, velusetrag, naronapride and the acetylcholinesterase inhibitor acotiamide, although their benefits are yet to be proven<sup>[12]</sup>.

Ghrelin is peptide hormone secreted by the gastric fundic mucosa and pancreas. It is the first identified circulating hormone that controls hunger. One important physiological action of ghrelin is regulation of gastric motility<sup>[32]</sup>. Intravenous use of the ghrelin agonist TZP-101 was reported to reduce nausea and vomiting

in patients with diabetic gastroparesis when compared to placebo<sup>[33]</sup>. Another study with the oral ghrelin analog TZP-102 also reported overall and individual reduction in the symptoms of diabetic gastroparetics<sup>[34]</sup>.

Some published case reports have also claimed efficacy for the dopamine antagonist thiethylpeazine, the neurokinin NK1 antagonist aprepitant and the antidepressant mirtazapine. A retrospective study reported decreased symptoms in 88% of diabetics with tricyclic antidepressants. The herbal extract STW5 (iberogast) is also reported to be beneficial in functional dyspepsia and gastroparesis<sup>[12]</sup>.

**Endoscopic and surgical treatments**

Mearin *et al*<sup>[35]</sup> proposed pyloric spasmodic contractions as one of the factors delaying gastric emptying. Endoscopic pyloric injections of botulinum toxin have been tried in the management of gastroparesis. This neurotoxin inhibits the release of acetylcholine at the neuromuscular junction, causing paralysis of the pylorus. Improved symptoms and accelerated gastric emptying persisting up to 3-6 mo were reported with pyloric botulinum toxin injections, especially in women and those with idiopathic gastroparesis<sup>[36]</sup>. It was also observed to be more beneficial in older men with vomiting<sup>[37]</sup>. However, small underpowered placebo-controlled trials did not show superior

responses for botulinum toxins *vs* placebo.

Gastric electrical stimulator implantations have also been shown to have benefits extending for more than 10 years and giving up to 80% reductions in nausea and vomiting. Additionally, there are reported improvements in nutritional and metabolic status, quality of life and health care utilizations<sup>[38]</sup>. Despite this, most studies show no effect on measured gastric emptying. One recent study showed improved symptoms in gastroparetics with gastric stimulators due to reduced gastric retention in diabetic patients<sup>[39]</sup>. Other newer technology in this field includes use of miniature wireless gastric stimulators inserted during endoscopy<sup>[40]</sup>. More studies are needed to ascertain the efficacy compared to other procedures.

Surgical treatments are rarely performed and are mainly reserved for patients with refractory gastroparetic symptoms who have failed to improve with other measures. A recent study demonstrated about 83% symptom reduction in gastroparetics after Heineke-Mikulicz pyloroplasty<sup>[41]</sup>. Completion gastrectomy was shown to give long-term symptom relief in some patients with post surgical gastroparesis, but data on patients with diabetic gastroparesis are limited. The possible benefits of pancreatic transplants for diabetic gastroparesis have not been proved<sup>[12]</sup>.

Other measures include jejunostomy feeding and total parenteral nutrition. Jejunostomy feeding improves overall health and shows trends towards reduced healthcare utilization in diabetic gastroparesis<sup>[42]</sup>. The role of venting percutaneous gastrostomy in refractory idiopathic gastroparesis is controversial. One study reported symptom improvement as well as improvement in nutritional and functional status in patients with idiopathic gastroparesis<sup>[43]</sup>. Total parenteral nutrition can reverse rapid weight loss and ensure adequate sustenance and is usually used in patients with associated intestinal dysmotility<sup>[12]</sup>.

## ENTEROPATHY

Small intestinal and colorectal dysfunctions are common in patients with longstanding diabetes, especially in those with gastroparesis<sup>[44]</sup>. Diabetes-related enteropathy may present with diarrhea, constipation or fecal incontinence. The mechanism of development of enteropathy is similar to that of upper GI involvement in diabetes<sup>[45]</sup>. Advanced glycation end products (AGEs) cause damage to cellular DNA and tissues in diabetes. AGEs and their receptors are increased in the ganglia, crypt and brush border of diabetic jejunum and ileum as well as in the ganglia of diabetic colon in animal models<sup>[46]</sup>. Damage to the myenteric nerve plexus due to autonomic neuropathy and fibrosis of the intestinal muscular layers result in stasis of the intestinal contents. Reduced bowel motility results in constipation that may sometimes lead to overflow incontinence. Small intestinal bacterial overgrowth (SIBO), which can result in diarrhea, is usually a consequence of intestinal stasis.

Constipation alternating with diarrhea is one of the most common symptoms of diabetic enteropathy. The

diarrhea is typically painless, may be associated with fecal incontinence and occurs during the day but more often at night<sup>[47]</sup>. Characteristically, it is seen in patients with poorly controlled diabetes who have peripheral and autonomic neuropathy<sup>[48]</sup>. Other causes of diarrhea in diabetics include pancreatic insufficiency, bile salt malabsorption, steatorrhea and drugs (Metformin). These should be excluded by appropriate investigations before making a diagnosis of diabetic enteropathy.

Constipation is a common problem affecting up to 60% of patients with long-standing diabetes mellitus<sup>[49]</sup>. Severe constipation leading to megacolon or colonic intestinal pseudo-obstruction occurs rarely. Stercoral ulcer, perforation and overflow diarrhea are encountered infrequently.

Fecal incontinence, particularly nocturnal, due to internal and external sphincter dysfunction secondary to autonomic neuropathy is a troublesome symptom. Acute hyperglycemia has been shown to inhibit external anal sphincter function and decrease rectal compliance, potentially increasing the risk of fecal incontinence<sup>[50]</sup>.

Patients should undergo endoscopic examination, ultrasound or computed tomography to exclude other diagnosis. Although aspiration and direct culture of jejunal contents are regarded by many as the gold standards for the diagnosis of SIBO<sup>[51]</sup>, these methods have several limitations, including the potential for contamination by oropharyngeal bacteria during intubation, and the fact that bacterial overgrowth may be patchy and may be missed by a single aspiration. Non-invasive diagnostic tests for SIBO are largely based on excretion of hydrogen in exhaled breath, following metabolism of carbohydrate by luminal bacteria. These tests have a specificity of 80%, but lack sensitivity (40%) and have their own limitations<sup>[52]</sup>. A radio opaque marker test is useful for excluding possible slow transit constipation. Tests for fecal incontinence include endoanal ultrasound and anorectal manometry.

Treatment of diabetic diarrhea mainly involves symptom relief, correction of fluid and electrolyte deficits, improvement of nutrition and glycemic control, and management of underlying causes<sup>[53]</sup>. Anti-diarrheal agents should be used with caution as there is a risk of toxic megacolon. Rifaximin is a minimally absorbed oral antimicrobial agent that is concentrated in the gastrointestinal tract, has broad spectrum *in vitro* activity against gram-positive and gram-negative aerobic and anaerobic bacteria, and has low risk of inducing bacterial resistance<sup>[54,55]</sup>. It has been shown to eradicate bacterial overgrowth in up to 84% of patients<sup>[56]</sup>. Other antibiotics used to treat this condition include amoxicillin-clavulanic acid, doxycycline, ciprofloxacin, metronidazole, neomycin and norfloxacin. There are anecdotal reports of successful treatment with somatostatin analogues of otherwise intractable secretory diarrhea in diabetic patients with autonomic neuropathy<sup>[57,58]</sup>.

Loperamide may prove useful in fecal incontinence. Constipation may be treated with prompt hydration, reg-

ular exercise and increased intake of dietary fibre. Lactulose and osmotic laxatives may be necessary in more severe cases. Newer drugs for treatment of chronic constipation include prucalopride, a selective 5-HT<sub>4</sub> receptor agonist that enhances colonic transit and lubiprostone, which stimulates colonic water and electrolyte secretion through activation of type 2 chloride channels in enterocytes. They may prove useful in the future for treatment of chronic constipation in diabetes mellitus due to autonomic neuropathy and slow transit.

## NONALCOHOLIC FATTY LIVER DISEASE

The definition of nonalcoholic fatty liver disease (NAFLD) requires that there is evidence of hepatic steatosis, either by imaging or by histology, and that there are no causes for secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication or hereditary disorders<sup>[59]</sup>. NAFLD is considered to be the hepatic manifestation of metabolic syndrome<sup>[60]</sup>. Metabolic syndrome encompasses the clinical tetrad of hyperinsulinemia with insulin resistance, visceral obesity, dyslipidemia and hypertension. In the majority of patients, NAFLD is associated with metabolic risk factors such as obesity (60%-95%), diabetes mellitus (28%-55%) and dyslipidemia (27%-92%) and, less clearly, with raised arterial pressure<sup>[61]</sup>. Histologically, NAFLD is further subdivided into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH).

Data from various studies indicate that the prevalence of NAFLD in the general population ranges from 6.3% to 33%. NAFLD is now the most common cause of chronic liver disease in North America, and it is estimated that 30% of the population of the United States has NAFLD<sup>[62]</sup>. In an ultrasonographic study, 69% of patients with T2DM had NAFLD<sup>[63]</sup>. Another study showed a prevalence of 62.3% (127 of the 204 diabetes patients had a fatty infiltration on ultrasound) and 87% of these patients with fatty infiltration who consented to biopsy had histological confirmation of the condition<sup>[64]</sup>.

### Clinical features, course and prognosis

Although the majority of patients with NAFLD are asymptomatic, some may present with nonspecific symptoms such as malaise and right upper quadrant pain. Clinical disease in NAFLD ranges from mild elevation of liver enzymes to severe liver disease with fibrosis and nodular degeneration. A recent study identified that approximately 30% of NAFLD cases with isolated steatosis will progress to NASH and, of these, approximately 20% will develop cirrhosis. About 40% of these cirrhotic patients develop decompensated liver disease<sup>[65]</sup>.

Patients with simple fatty change had no increase in mortality, whereas patients with NASH had reduced survival and more cases died from cardiovascular disease (15.5% *vs* 7.5%) than liver related disease (2.8% *vs* 0.2%)<sup>[66]</sup>. Another long term study, conducted in Minnesota United States, of 420 patients in the community

with NAFLD showed higher mortality in patients with impaired fasting glucose and cirrhosis, when compared with the general population. Liver-related mortality was also higher in this group than in the general population (13% *vs* < 1%)<sup>[67]</sup>.

Hepatocellular carcinoma (HCC) is a well recognized complication of cirrhosis due to NAFLD<sup>[68-71]</sup>. Diabetes, obesity and cirrhosis-associated carcinogenic factors may have roles in the development of HCC in patients with NAFLD<sup>[68,70,72]</sup>. Presence of diabetes, elevated body mass index and liver fibrosis were identified as risk factors for progression to HCC among NAFLD cases<sup>[73]</sup>. Recent evidence from animal models shows that metabolic syndrome itself is high risk state for the development of NASH and HCC<sup>[74]</sup>.

### Pathogenesis

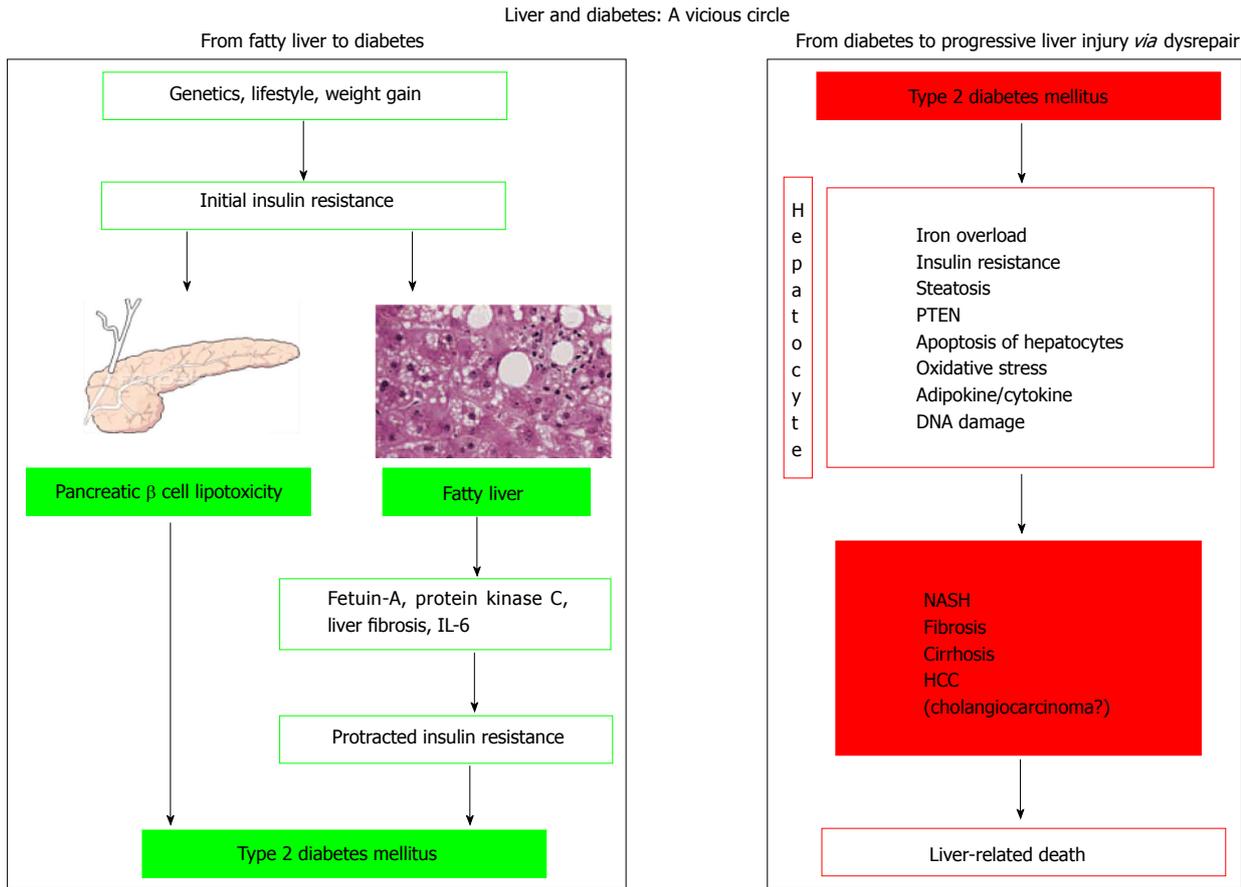
The development of NAFLD involves complex mechanisms and the relationship between T2DM and NAFLD is depicted in the Figure 1. Obesity, insulin resistance and metabolic syndrome are linked to the development of NAFLD<sup>[75]</sup>. It is now postulated that a combination of "multi hits" leads to development of steatohepatitis. This concept has replaced the earlier two hit hypothesis<sup>[76,77]</sup>. There is strong association between NASH, insulin resistance and increased level of free fatty acids in the liver<sup>[78,79]</sup>. Several factors including tumor necrosis factor alpha, oxidative stress, adiponectin, leptin, apoptosis and genetic factors are believed to have a role in the pathogenesis of NAFLD and NASH.

### Evaluation

According to guidelines from the American Association for the Study of Liver Diseases (AASLD), the diagnosis of NAFLD requires that there is hepatic steatosis by imaging or histology, there is no significant alcohol overconsumption, there are no competing etiologies for hepatic steatosis, and there are no co-existing causes for chronic liver disease<sup>[59]</sup>.

The following conditions should be excluded: history of alcohol intake > 20 g/d, nutritional causes (*e.g.*, total parenteral nutrition and rapid weight loss), metabolic disorders (glycogen storage disorders), chronic hepatitis C (particularly genotype 3), other causes of chronic liver diseases (autoimmune liver disease, Wilson's disease and hemochromatosis) and endocrine disorders such as polycystic ovary syndrome, hypopituitarism and hypothyroidism. Drug-induced steatosis can be caused by a number of agents including glucocorticoids, synthetic estrogens, amiodarone, methotrexate and highly active antiretroviral drugs. NAFL is considered benign whereas NASH can progress to cirrhosis, liver failure, and liver cancer.

Liver biopsy is considered the most reliable approach for identifying the presence of steatohepatitis and fibrosis in patients with NAFLD, but its limitations include cost, sampling error, and procedure-related morbidity and mortality. Features of the metabolic syndrome can predict the presence of steatohepatitis in patients with



**Figure 1** Molecular mechanisms involved in the vicious circle linking fatty liver to diabetes and diabetes to progressive liver injury. Left: The first part of the journey, leading from initial insulin resistance to fatty liver and eventually to the development of type 2 diabetes mellitus (T2DM) in those predisposed individuals in whom pancreatic lipotoxicity occurs; Right: The mechanism that (triggered by long-lasting/decompensated T2DM) may be conducive to progressive liver disease including primary liver cancer in predisposed individuals. HCC: Hepatocellular carcinoma; IL: Interleukin; NASH: Non-alcoholic steatohepatitis; PTEN: Phosphatase and tensin homolog. Reproduced from Loria *et al*<sup>[121]</sup>.

NAFLD. Hence, liver biopsy is recommended in patients with NAFLD who have the metabolic syndrome<sup>[80-84]</sup>. There has been increasing interest in developing non-invasive methods to identify fibrosis in patients with NAFLD. NAFLD Fibrosis Score is a clinically useful tool for identifying NAFLD patients with higher likelihood of having bridging fibrosis and/or cirrhosis. In a meta-analysis of 13 studies consisting of 3064 patients, it was shown that NAFLD Fibrosis Score has a 90% sensitivity and 60% specificity to exclude advanced fibrosis and 67% sensitivity and 97% specificity to identify the presence of advanced fibrosis<sup>[83]</sup>. The NAFLD Fibrosis Score is based on six variables [age, body mass index (BMI), hyperglycemia, platelet count, albumin, aspartate aminotransferase/alanine aminotransferase ratio] and it is calculated using the published formula (<http://naflscore.com>).

A novel biomarker that has been investigated for the presence of steatohepatitis in patients with NAFLD is circulating levels of cytokeratin-18 fragments<sup>[85,86]</sup>. This has a sensitivity of 78% and specificity of 87% for identifying steatohepatitis in patients with NAFLD. Transient elastography (TE), which measures liver stiffness non-invasively, showed high sensitivity and specificity for iden-

tifying fibrosis in NAFLD in a recent meta-analysis<sup>[85]</sup>. However, TE has a high failure rate in individuals with a higher BMI. There is some evidence that the Enhanced Liver Fibrosis test which uses the fibrosis markers hyaluronic acid, amino-terminal propeptide-of-type-III-collagen and tissue-inhibitor of matrix-metalloproteinase-1, compares favourably with the use of TE<sup>[87]</sup>.

**Management**

The management of patients with NAFLD consists of treating liver disease and the associated metabolic comorbidities such as obesity, hyperlipidemia, insulin resistance and T2DM.

**Lifestyle modification and weight reduction:** Modifications in diet and lifestyle along with weight reduction and exercise are the cornerstones of treatment of NAFLD, as it is a disease related to excess weight and sedentary lifestyle. Many studies have shown that lifestyle modification can reduce aminotransferase levels and improve hepatic steatosis when measured either by ultrasound<sup>[88-91]</sup> or MR imaging and spectroscopy<sup>[92-95]</sup>. A randomized study of 31 obese persons with NASH who

underwent intensive lifestyle changes (diet, behaviour modification and 200 min a week of moderate physical activity for 48 wk) *vs* structured basic education alone showed improvement in steatosis, necrosis and inflammation in the obese group and participants with 7% weight loss had significant improvement in steatosis, lobular inflammation, ballooning, and NAFLD Activity Score<sup>[96]</sup>.

**Insulin sensitizing agents:** Insulin resistance plays a key role in the pathogenesis of NAFLD. The two main classes of insulin-sensitizing drugs used in the management of patients with NAFLD/NASH are biguanides (metformin) and the thiazolidinediones (pioglitazone).

Metformin increases insulin sensitivity by decreasing hepatic gluconeogenesis and decreasing triglyceride production<sup>[97]</sup>. Early small, open-label studies showed a reduction in insulin resistance and serum levels of aminotransferases<sup>[98-100]</sup> but no significant improvement in liver histology<sup>[99,100]</sup>. A recent meta-analysis examining effects of medical treatment and/or lifestyle intervention did not show significant benefit of metformin in NAFLD<sup>[101]</sup>. Metformin showed no effect on liver histology and is not recommended as a specific treatment for liver disease in adults with NASH.

Pioglitazone has been available for over a decade for the treatment of T2DM. It acts by promoting peripheral and hepatic insulin sensitivity and increasing circulating levels of adiponectin<sup>[102]</sup>. A recent meta-analysis showed that pioglitazone improved histological disease activity, glucose, lipid and inflammatory variables and delayed fibrosis progression in patients with NAFLD<sup>[101]</sup>. The current recommendation by AASLD is that Pioglitazone can be used to treat steatohepatitis in patients with biopsy-proven NASH, although the long term safety and efficacy of pioglitazone in patients with NASH is unknown.

**Vitamin E:** The antioxidants vitamin E and betaine were investigated as potential therapeutic agents in NASH<sup>[103,104]</sup>. When administered for 2 years vitamin E improved liver histology, but increased insulin resistance and plasma triacylglycerols<sup>[101]</sup>. Therefore, the current recommendation by the AASLD is that vitamin E ( $\alpha$ -tocopherol), administered at a daily dose of 800 IU/d should be considered as first-line pharmacotherapy for non-diabetic adults with biopsy-proven NASH. However, vitamin E is not recommended to treat NASH in diabetic patients, NAFLD without liver biopsy, NASH cirrhosis or cryptogenic cirrhosis due to lack of supporting evidence.

**Incretin mimetics:** Incretins are a group of gastrointestinal hormones released after food intake that enhance insulin release from pancreatic beta cells. The most studied among these hormones is GLP-1. The role of the GLP-1 analogues exenatide and liraglutide in the management of T2DM in obesity is well established. These drugs may emerge as new options in management of NAFLD because of similar mechanisms in its pathogenesis.

Dipeptidyl-peptidase IV (DPP4) inhibitors were introduced as an alternative means to increase GLP-1 activity. There is increased serum DPP4 activity in patients with NASH, and this has a positive correlation with the histological grade and degree of liver steatosis<sup>[105]</sup>. DPP4 inhibitors are already established oral treatments for type 2 diabetes<sup>[106]</sup>, and data from experimental studies suggest that they may also reduce liver inflammation and steatosis<sup>[107]</sup>. Incretin mimetics may, in the future, represent a novel therapeutic option for slowing the progression of NAFLD.

**Omega-3 fatty acids:** So far, there is no clear evidence for the use of omega-3 fatty acids for the specific treatment NAFLD and NASH<sup>[108]</sup>. A large multicenter study of omega-3 fatty acid (eicosapentanoic acid) for treatment of NASH is ongoing in the United States.

**Other agents:** Orlistat, Sibutramine and Rimonabant (a cannabinoid receptor antagonist) have all been investigated for their potential as weight loss medications in NAFLD/NASH, although Sibutramine and Rimonabant have been withdrawn due to their side effects<sup>[109]</sup>. A single large multicenter randomized controlled trial showed that ursodeoxycholic acid offers no histological benefit over placebo in patients with NASH<sup>[110]</sup>. Recent data from animal models showed that consumption of hydrogen-rich water may be an effective treatment for NASH by reducing hepatic oxidative stress, apoptosis, inflammation, and hepatocarcinogenesis<sup>[111]</sup>.

**Bariatric surgery:** AASLD recommends that foregut bariatric surgery is not contraindicated in otherwise eligible obese individuals with NAFLD or NASH. In a study of 381 adult obese patients by Mathurin *et al*<sup>[112]</sup> there was a significant improvement in the prevalence and severity of steatosis and ballooning at 1 and 5 years following bariatric surgery. A recently published Cochrane review concluded that lack of randomized clinical trials or quasi-randomized clinical studies precludes definitive assessment of the benefits and harms of bariatric surgery as a therapeutic approach for patients with NASH<sup>[113]</sup>.

## GLYCOGENIC HEPATOPATHY

Glycogenic hepatopathy is defined as pathological overloading of hepatocytes with glycogen leading to hepatic enlargement and/or derangement of liver enzymes and is usually seen in patients with longstanding poorly-controlled type 1 diabetes mellitus (T1DM)<sup>[114]</sup>. Glycogen accumulation in the liver was first described in 1930 as a component of Mauriac's Syndrome. This syndrome was characterized by unstable diabetes, hepatomegaly, hyperlipidemia, dwarfism, cushingoid features and delayed sexual maturity. It is now recognized that glycogen accumulation within hepatocytes can be present without all the findings described in Mauriac's Syndrome. Inadequate

control of T1DM results in concomitant presence of insulin and excess glucose that increases glycogen storage in the liver. Insulin activates the enzyme glycogen synthase phosphatase which dephosphorylates and activates glycogen synthase, another enzyme that is required for the conversion of glucose-1-phosphate to glycogen<sup>[115]</sup>. This results in increased glycogen storage in the liver and blocks glycogenolysis. The histological picture is characterized by pale appearance of the hepatocytes with compression of the sinusoids, glycogenated nuclei and giant mitochondria. Steatosis may be present, usually mild, or absent. Glycogen accumulation, the hallmark of this condition is demonstrated by PAS-diastase staining<sup>[114]</sup>.

The disease is under-recognized and usually presents with abdominal pain, nausea, vomiting and abnormalities in liver function tests. While hepatic dysfunction is usually due to NAFLD in T2DM, liver dysfunction in T1DM usually results from glycogenic hepatopathy. It cannot be distinguished from NAFLD clinically or by ultrasound and confirmation requires a liver biopsy. The disorder should be suspected when liver dysfunction occurs in patients with T1DM, especially when viral, autoimmune and metabolic liver diseases are excluded by laboratory investigations. The hallmark of this condition is its reversibility with improved glycemic control. Unlike hepatic steatosis, glycogen overload is not known to progress to fibrosis distinct from fatty liver disease<sup>[116]</sup>. Prompt improvement with optimal diabetes control by insulin treatment within 4 wk is usually seen in these patients<sup>[117,118]</sup>.

## HEPATOGENOUS DIABETES

Up to 79% of cirrhotic subjects can have abnormalities of glucose metabolism<sup>[119]</sup>. T2DM is usually associated with metabolic syndrome that can lead to NAFLD and cirrhosis. The term “hepatogenous diabetes” (HD) is used to describe diabetes developing in patients with cirrhosis<sup>[119]</sup>. Numerous factors, including reduced insulin clearance, peripheral hyperinsulinemia and down-regulation of insulin receptors, lead to development of diabetes in cirrhosis<sup>[120]</sup>. HD is clinically different from T2DM in that it is less frequently associated with microangiopathy and patients suffer from complications of cirrhosis more frequently. However, HD is not yet recognized by the American Diabetes Association and the World Health Organization.

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## Cardiometabolic health among gastric bypass surgery patients with polycystic ovarian syndrome

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### Abstract

**AIM:** To examine the effect of gastric bypass surgery on cardiometabolic health among women with polycystic ovarian syndrome (PCOS).

**METHODS:** Retrospective medical chart review identified women ( $n = 389$ ) with PCOS who underwent Roux-en-Y gastric bypass surgery from 2001-2009 in one surgical practice. Separate repeated measures linear mixed models were fit using the MIXED procedure

to assess mean change in cardiometabolic disease risk factors from before to 1-year after surgery and were evaluated by ethnicity [Hispanic, non-Hispanic black (NHB) and white (NHW)].

**RESULTS:** The majority of the sample was Hispanic (66%, 25% NHB, 9% NHW). Mean body mass index significantly improved 1 year post-surgery for all ethnic groups (45.5 to 35.5 kg/m<sup>2</sup> for Hispanics, 46.8 to 37.7 kg/m<sup>2</sup> for NHB and 45.7 to 36.7 kg/m<sup>2</sup> for NHW,  $P < 0.001$ ). Among Hispanic women mean total cholesterol (198.1 to 160.2 mg/dL), low-density lipoproteins (LDL) cholesterol (120.9 to 91.0 mg/dL), triglycerides (148.6 to 104.8 mg/dL), hemoglobin A1c (6.2% to 5.6%), alanine aminotransferase (28.1 to 23.0 U/L) and aspartate aminotransferase (23.5 to 21.6 U/L) decreased significantly ( $P < 0.001$ ). Among NHB, mean total cholesterol (184.5 to 154.7 mg/dL), LDL cholesterol (111.7 to 88.9 mg/dL) and triglycerides (99.7 to 70.0 mg/dL) decreased significantly ( $P < 0.05$ ). Among NHW, mean total cholesterol (200.9 to 172.8 mg/dL) and LDL cholesterol (124.2 to 96.6 mg/dL), decreased significantly ( $P < 0.05$ ). Pair-wise ethnic group comparisons of all cardiometabolic outcomes adjusted for age and type of surgery before and 1 year after surgery showed no statistical difference between the three groups for any outcome.

**CONCLUSION:** Cardiometabolic disease risk improvements vary by ethnicity and obesity may impact glucose tolerance and liver function changes more in Hispanic women with PCOS vs non-Hispanic women.

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**Key words:** Bariatric surgery; Polycystic ovary syndrome; Cardiometabolic outcomes; Weight outcomes; Morbid obesity

**Core tip:** Morbidly obese women with polycystic ovary syndrome (PCOS) who have undergone Roux-en-Y

gastric bypass surgery show improvement in cardiometabolic disease risk at one year after the procedure. All women demonstrated a decrease in cardiometabolic disease risk factors including body mass index, glycated hemoglobin A1c (HbA1c), alanine aminotransferase (ALT), total cholesterol (TC), low-density lipoproteins (LDL) and triglyceride (TG). However, only Hispanic women showed a significant decreasing HgA1c and ALT, TC, LDL and TG one year after surgery. These ethnic group differences suggest there may be more comprehensive benefits in terms of cardiometabolic disease risk reduction after bariatric surgery in Hispanic women with PCOS vs other ethnic groups.

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## INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complex condition strongly associated with obesity, diabetes, coronary heart disease, and certain cancers<sup>[1-3]</sup>. Often presents in adolescents and is probably the most common endocrinopathy in women. PCOS affects 5%-10% of women of reproductive age and up to 28% of overweight or obese women<sup>[4-6]</sup>. Diagnostic features consist of clinical evidence of anovulation and clinical or biochemical evidence of hyperandrogenism after exclusion of other etiologies of hyperandrogenism. Consequently, it is the most common cause of anovulatory infertility, oligomenorrhoea, amenorrhoea, and hirsutism<sup>[7]</sup>.

It is widely reported that women with PCOS have a higher prevalence of cardiometabolic disease risk factors and overt disease compared to non-PCOS women<sup>[7]</sup>. Specifically, obesity, insulin resistance, metabolic syndrome, diabetes, dyslipidemia and nonalcoholic fatty liver disease (NAFLD) are commonly seen in women with PCOS<sup>[7]</sup>. Limited studies have shown that weight loss after bariatric surgery in women with PCOS results in improvement or elimination of the symptoms of PCOS and associated cardiometabolic risk factors<sup>[8-10]</sup>. For example, women with PCOS following surgery have demonstrated normalization of menses, hirsutism score, serum androgens, and insulin sensitivity. However, it is less clear how effective bariatric surgery is on the resolution of pre-diabetes, hypertension and dyslipidemia in affected women with PCOS following bariatric surgery, and in multiethnic women in particular. Hispanics have a higher prevalence of obesity, impaired glucose tolerance, type 2 diabetes and NAFLD compared to their NHW counterparts, and consequently a higher risk for subsequent cardiovascular disease<sup>[11-13]</sup>. Therefore, weight loss in obese Hispanic women with PCOS may

improve the risk of diabetes more substantially compared to non-Hispanic black (NHB) and NHW subjects but these comparisons are largely unknown. The objective of this analysis was to examine the effect of weight loss after bariatric surgery on cardiometabolic disease risk factors in a primarily Hispanic population of women with PCOS.

## MATERIALS AND METHODS

A retrospective medical chart review identified women with a history or diagnosis of PCOS who underwent Roux-en-Y gastric bypass surgery ( $n = 389$ ) in one private practice from 2001-2009. Diagnosis of PCOS was self reported and confirmed by an endocrinologist or gynecologist. Pre-surgery and 12 mo post-surgical cardiometabolic risk factors and ethnicity were collected for comparative analysis. Cardiometabolic risk factors included weight, height, body mass index (BMI), total cholesterol (TC), high- and low-density lipoproteins (HDL and LDL), triglyceride (TG), glycated hemoglobin A1c (HbA1c), and alanine aminotransferase (ALT) and aspartate aminotransferase (AST). This study was approved by and in accordance with the ethical standards of the Institutional Review Board.

### Data collection

We abstracted data collected before and 1 year after surgery. In addition to data on sex, race, and ethnicity, these data included BMI and concentrations of HbA1c, HDL, LDL, TC, TG, AST and ALT. Pre-operative comorbidities and surgical complications were also recorded.

### Measurements

Height and weight were measured during routine clinical visits by practice nursing staff. Weight was measured to the nearest 0.1 pound (and converted to kilograms for analyses) using a digital scale with the participants wearing light clothing and no shoes. Height was measured to the nearest 0.5 cm using an Accustat Genentech stadiometer. Body mass index was calculated as body weight in kilograms divided by height in meters squared ( $\text{kg}/\text{m}^2$ ). Excess weight loss (EWL) was calculated as show in the following equation:  $\text{EWL} (\%) = (\text{Preoperative weight} - \text{follow up weight}) / (\text{Preoperative weight} - \text{ideal body weight}) \times 100\%$ .

Ideal body weight was calculated in base of the 1983 Metropolitan Life Insurance tables for gender and height. All blood samples were collected, processed, stored at  $-20^\circ\text{C}$ , and shipped to the laboratory for analysis. HbA1c was analyzed by turbidimetric immunoinhibition with commercially available reagents. TC, LDL, HDL and TG were analyzed enzymatically with commercially available reagents. ALT and AST were determined by kinetic-spectrophotometric technique.

### Surgery criteria

All patients met the National Institutes of Health criteria for bariatric surgery<sup>[14]</sup>. Thus, all patients had a BMI

**Table 1 Mean body mass index and cardiometabolic disease risk factor values among women with polycystic ovary syndrome before and 1 year after gastric bypass surgery by ethnic group**

	<i>n</i> <sup>4</sup>	Pre-surgery Estimate (95%CI)	1-yr follow up Estimate (95%CI)
<b>Hispanic (<i>n</i> = 257)<sup>1</sup></b>			
BMI (kg/m <sup>2</sup> )	257	45.5 (44.7-46.4)	35.5 (34.6-36.3)
Weight (kg)	257	118.4 (115.8-120.9)	92.3 (89.8-94.7)
EWL (%)	257		54.5 (1.9)
Percent weight loss (%)	257		22.0 (0.6)
Total cholesterol (mg/dL)	28	198.1 (190.4-205.8)	160.2 (154.0-166.4)
HDL cholesterol (mg/dL)	28	49.5 (46.8-52.1)	48.7 (46.4-51.0)
LDL cholesterol (mg/dL)	24	120.9 (112.9-128.9)	91.0 (86.0-95.9)
Triglycerides (mg/dL)	28	148.6 (128.7-168.6)	104.8 (93.9-115.8)
Hemoglobin A1c (%)	58	6.2 (6.0-6.3)	5.6 (5.5-5.6)
ALT (U/L)	99	28.1 (25.8-30.4)	23.0 (20.7-25.4)
AST (U/L)	100	23.5 (22.0-25.0)	21.6 (19.8-23.4)
<b>Non-Hispanic black (<i>n</i> = 97)<sup>2</sup></b>			
BMI (kg/m <sup>2</sup> )	97	46.8 (45.5-48.2)	37.7 (36.2-39.2)
Weight (kg)	97	128.2 (123.5-132.8)	103.1 (98.5-107.8)
EWL (%)	97		47.5 (3.1)
Percent weight loss (%)	97		19.5 (1.0)
Total cholesterol (mg/dL)	6	184.5 (166.6-202.3)	154.7 (140.2-169.2)
HDL cholesterol (mg/dL)	6	50.6 (45.0-56.1)	53.0 (47.4-58.6)
LDL cholesterol (mg/dL)	6	111.7 (97.0-126.5)	88.9 (76.4-101.3)
Triglycerides (mg/dL)	6	99.7 (80.0-119.5)	70.0 (56.8-83.3)
Hemoglobin A1c (%)	15	6.4 (5.9-6.8)	5.8 (5.3-6.3)
ALT (U/L)	23	18.6 (16.3-20.9)	16.0 (12.6-19.4)
AST (U/L)	23	19.2 (17.4-20.9)	18.6 (16.4-20.7)
<b>Non-Hispanic white (<i>n</i> = 35)<sup>3</sup></b>			
BMI (kg/m <sup>2</sup> )	35	45.7 (43.2-48.1)	36.7 (34.4-39.0)
Weight (kg)	35	123.9 (116.5-131.2)	99.58 (92.3-106.9)
EWL (%)	35		50.2 (5.2)
Percent weight loss (%)	35		20.2 (1.7)
Total cholesterol (mg/dL)	7	200.9 (178.1-223.7)	172.8 (160.5-185.2)
HDL cholesterol (mg/dL)	7	49.7 (44.3-55.1)	49.6 (43.8-55.4)
LDL cholesterol (mg/dL)	6	124.2 (104.2-144.2)	96.6 (80.4-112.9)
Triglycerides (mg/dL)	7	160.5 (120.9-200.1)	137.8 (104.9-170.7)
Hemoglobin A1c (%)	5	6.3 (5.8-6.8)	5.9 (5.0-6.8)
ALT (U/L)	10	30.0 (23.7-36.0)	22.0 (16.6-27.4)
AST (U/L)	10	23.7 (19.7-27.8)	20.8 (16.6-25.0)

<sup>1</sup>Differences were statistically significant ( $P < 0.001$ ), except for high-density lipoprotein (HDL) cholesterol and aspartate aminotransferase (AST);

<sup>2</sup>All differences were statistically significant ( $P < 0.05$ ), except for HDL cholesterol, hemoglobin A1c (HbA1c), alanine aminotransferase (ALT) and AST were not significant; <sup>3</sup>All differences were statistically significant ( $P < 0.05$ ) except for HDL cholesterol, triglycerides, HbA1c, ALT and AST;

<sup>4</sup>Number of patients with data at baseline and 1 year follow up. BMI: Body mass index; EWL: Estimated weight loss; LDL: Low-density lipoprotein.

greater than 35 kg/m<sup>2</sup> and at least one other risk factor for cardiometabolic disease (*e.g.*, elevated blood pressure, hypercholesterolemia, and so on) or a BMI greater than 40 kg/m<sup>2</sup>. Patients received group and individual education from an interdisciplinary group of providers about surgical and nonsurgical treatments, outcomes, complications, and necessary lifestyle changes. All patients underwent psychological and nutritional evaluation and testing before surgery.

### Surgical procedure

All patients underwent Roux-en-Y gastric bypass surgery. The majority of patients spent one night in the hospital

after surgery. All patients were scheduled for routine post-operative visits at 1, 3, 6 and 12 mo post-surgery.

Patients had a Roux limb length of either 125 or 150 cm, depending on the patient's baseline BMI. The Roux limb was brought up in an antecolic, antegastric manner. The jejunal-jejunal mesenteric defect was closed with a permanent running suture. The entire gastrojejunostomy was over-sewn with a circumferential permanent running suture. All patients went to the bariatric floor postoperatively and were started on liquids after a normal upper gastrointestinal study on post-operative day 1. All patients were discharged home on a standard bariatric liquid diet for 1 wk.

### Statistical analysis

Separate repeated measures linear mixed models were fit using the MIXED procedure to assess change all cardiometabolic disease risk factors (TC, HDL, LDL, BMI, HbA1c, AST and ALT) before and 1-year after bariatric surgery were evaluated by ethnicity (Hispanic, NHB and NHW).

## RESULTS

The majority of the sample was Hispanic ( $n = 257$ , 66%), 25% ( $n = 97$ ) were NHB and 9% ( $n = 35$ ) were NHW; mean age was 40.9 years, (SD 12.9, range 16-74 years). BMI significantly improved from before to 1 year after surgery for all ethnic groups ( $P < 0.0001$ ). Among Hispanic women all before-after differences in cardiometabolic disease risk factors were statistically significant ( $P < 0.001$ ) with the exception of HDL cholesterol and AST (not significant). Among NHB, HDL Cholesterol, HbA1c, ALT and AST did not decrease significantly 1-year after surgery. Among NHW, all differences were statistically significant ( $P < 0.05$ ), except for HDL cholesterol, triglycerides, HbA1c and AST (not significant) (Table 1). Pairwise ethnic group comparisons adjusted for age and type of surgery of before and 1 year after surgery cardiometabolic outcomes showed no statistical difference between the three groups for any outcome (Table 2).

### Limitations

Our study has some limitations. In the evaluation of hepatic steatosis, we were not able to evaluate liver ultrasound or liver biopsy prior to and after bariatric surgery which may be more specific to detect pathology. Blood pressures were not available in all patients to assess hypertension as an additional cardiovascular disease risk factor. Finally, we were unable to assess potential confounders such as lifestyle factors (physical activity and diet) because this information was not assessed in patients.

## DISCUSSION

This analysis shows that Roux-en-Y gastric bypass surgery results in a significant decrease in BMI among Hispanic, NHWs and NHBs with a history or diagnosis

**Table 2** Ethnic group comparison of change in mean cardiometabolic disease risk factors among women with polycystic ovary syndrome 12 mo, after gastric bypass surgery performed between 2001 and 2010

Anthropometric measures	Group	Estimated change 12 mo after surgery (SE)	P value	P value Ethnic group comparisons	
				NHB	NHW
BMI (kg/m <sup>2</sup> )	Overall	9.7 (0.3)	< 0.0001	NHB	NHW
	Hispanic	10.1 (0.3)	< 0.0001	0.09	0.19
	NHB	9.1 (0.5)	< 0.0001	-	0.87
	NHW	9.0 (0.8)	< 0.0001	0.87	-
Weight (kg)	Overall	25.7 (0.7)	< 0.0001	NHB	NHW
	Hispanic	26.1 (0.8)	< 0.0001	0.49	0.40
	NHB	25.0 (1.3)	< 0.0001	-	0.76
	NHW	24.3 (2.0)	< 0.0001	0.76	-
Total cholesterol (mg/dL)	Overall	35.1 (3.6)	< 0.0001	NHB	NHW
	Hispanic	37.9 (4.2)	< 0.0001	0.36	0.21
	NHB	29.7 (7.8)	0.01	-	0.63
	NHW	28.1 (10.8)	0.04	0.63	-
HDL cholesterol (mg/dL)	Overall	-0.1 (1.2)	0.95	NHB	NHW
	Hispanic	0.7 (1.5)	0.64	0.26	0.96
	NHB	-2.4 (2.6)	0.40	-	0.36
	NHW	0.1 (2.9)	0.98	0.36	-
LDL cholesterol (mg/dL)	Overall	27.8 (3.4)	< 0.0001	NHB	NHW
	Hispanic	29.9 (4.3)	< 0.0001	0.32	0.89
	NHB	22.9 (6.2)	0.01	-	0.51
	NHW	27.6 (7.3)	0.01	0.51	-
Triglycerides (mg/dL)	Overall	40.1 (7.6)	< 0.0001	NHB	NHW
	Hispanic	43.8 (10.4)	0.0003	0.36	0.26
	NHB	29.7 (7.5)	0.01	-	0.64
	NHW	22.7 (12.8)	0.13	0.64	-
Hemoglobin A1c (%)	Overall	0.6 (0.1)	< 0.0001	NHB	NHW
	Hispanic	0.6 (0.1)	< 0.0001	0.77	0.38
	NHB	0.5 (0.3)	0.11	-	0.66
	NHW	0.37 (0.3)	0.31	0.66	-
ALT (U/L)	Overall	4.9 (1.1)	< 0.0001	NHB	NHW
	Hispanic	5.1 (1.3)	0.0002	0.16	0.38
	NHB	2.6 (1.4)	0.08	-	0.16
	NHW	7.9 (3.7)	0.06	0.16	-
AST (U/L)	Overall	1.8 (0.8)	0.04	NHB	NHW
	Hispanic	1.9 (1.1)	0.07	0.37	0.66
	NHB	0.6 (1.1)	0.56	-	0.40
	NHW	2.9 (2.8)	0.33	0.40	-

NHB: Non-Hispanic black; NHW: Non-Hispanic white; BMI: Body mass index; EWL: Estimated weight loss; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

of PCOS. Several ethnic group differences in pre-post change in individual cardiometabolic disease risk factors were found. Specifically, while all ethnic groups showed improvement in HbA1c and ALT, the decrease was significant only in Hispanic subjects. Additionally, all ethnic groups showed improvement in TC, LDL and TG one year after surgery. However, improvement in TC and TG were only significant for Hispanics and NHB subjects. Decrease in LDL was only significant in Hispanic and NHW subjects.

Our results are similar to previous studies of much smaller sample sizes that have shown that weight loss after bariatric surgery improves cardiometabolic disease risk among women with PCOS including insulin resistance and dyslipidemia, as well as biochemical features we did

not include such as hyperandrogenism and oligomenorrhea<sup>[8-10]</sup>. Specifically, Escobar-Morreale *et al*<sup>[8]</sup> followed 12 Caucasian women of European ancestry with PCOS after bariatric surgery and found significant improvements in hirsutism and normalization of serum androgens in 91% of subjects at follow up (mean 12 mo, range 7-26 mo). All patients had regular menses after weight loss and 10 subjects had confirmed ovulation. They also found normalization of insulin sensitivity based on homeostasis model assessment (HOMA) in all patients. Similarly, Eid *et al*<sup>[9]</sup> followed 24 American women, whom were primarily Caucasian, with PCOS after Roux-en-Y gastric bypass (mean follow up 27 mo, range 12-57 mo). All subjects had normalization of menses and 77% had significant improvement or resolution of hirsutism. Eleven of the 24 subjects had type 2 diabetes prior to surgery and all 11 were normoglycemic at follow up without glucose lowering medications. Likewise, eleven patients with dyslipidemia prior to surgery no longer required cholesterol lowering medications at follow up. The most recent study by Jamal *et al* followed 20 American women with PCOS after Roux-en-Y gastric bypass (mean follow up 46.7 mo, range 15-123 mo)<sup>[10]</sup>. They found similar improvements in menses, hirsutism and type 2 diabetes. Six patients that desired pregnancy were able to conceive. These studies indicate weight loss through bariatric surgery significantly improved symptoms of PCOS and associated cardiometabolic disease risk and confirm our findings.

Hispanic ethnicity is associated with an increased cardiometabolic disease risk. In the Diabetes Prevention Trial, Hispanic adults with impaired glucose tolerance were found to have a higher HbA1c and HOMA after adjusting for BMI compared with white subjects with similar fasting plasma glucose levels<sup>[15]</sup>. Similarly, in the Nurse's Health Study the relative risk of type 2 diabetes among Hispanic women was 2.18 compared to white women after adjusting for BMI<sup>[13]</sup>. In addition, relative risk for type 2 diabetes is higher in Hispanics than in African-Americans<sup>[13]</sup>. Obesity further amplifies this risk; it has been reported that for every 5 point increase in BMI the relative risk for diabetes in Hispanic women is 2.36 *vs* 1.96 in NHW women<sup>[13]</sup>. Furthermore, obese Hispanic women are at a higher risk of diabetes compared to NHW and NHB women. Therefore, weight loss in obese Hispanic women with PCOS may improve the risk for overt diabetes more substantially compared to NHB and NHW subjects. Our results here showed significant ethnic group differences in the post-surgery improvement in HbA1c. While HbA1c improved in all groups, the improvement was only significant among Hispanic women with PCOS. This specific finding suggests that obesity may impact glucose tolerance more in Hispanic women with PCOS *vs* non-Hispanic women.

Hispanics also have a higher prevalence of NAFLD which is associated with obesity, insulin resistance and increased metabolic risk<sup>[11]</sup>. NAFLD represents a spectrum of fat accumulation in the liver from steatosis to cirrhosis and is the most common cause of elevated liver

enzymes<sup>[16]</sup>. The estimated United States prevalence of NAFLD is approximately 25% in the general population, but has been found to be as high as 45% in some Hispanic populations *vs* 33% among NHW and 25% among NHB<sup>[11,17]</sup>. The prevalence of hepatic steatosis on ultrasound among NHW women with PCOS is 55% and may be as high as 90% in some obese cohorts<sup>[17,18]</sup>. Hispanic women with PCOS also had a higher prevalence of NAFLD compared to Caucasian subjects<sup>[11,12]</sup>. Women with PCOS have a low prevalence of elevated transaminases despite radiographic evidence of hepatic steatosis. Gambarin-Gelwan *et al*<sup>[18]</sup> determined that only 15% of women with PCOS and steatosis on ultrasound had abnormal liver enzymes. We found in our population of women with PCOS that Hispanic and NHW subjects had a higher baseline ALT compared to NHB subjects. Post-surgery, Hispanic women with PCOS had a significant decrease ALT, whereas the decrease in ALT in NHW and NHB subjects was not significant. This decreased in ALT in the Hispanic population may indicate improvement of fatty infiltration after surgery and therefore, weight loss in obese Hispanic women with PCOS may have a greater impact on hepatic steatosis compared to NHW and NHB subjects.

We also found differential improvements in cholesterol among the Hispanic and NHB subjects. The majority of American women with PCOS have a lipid abnormality<sup>[19]</sup>. Most display an atherogenic lipid profile including high triglyceride concentrations, high LDL and low HDL<sup>[20]</sup>. Browning *et al*<sup>[11]</sup> found in a sample of 12 women the majority with PCOS that required cholesterol lowering medication prior to surgery were able to discontinue medication at follow up based on improvements in total cholesterol and triglycerides. We found that all ethnic groups showed improvement in total cholesterol, LDL and TG after surgery. However, total cholesterol and triglycerides showed significant improvement only for Hispanics and NHB subjects, and improvement in LDL was only significant for Hispanics and NHW subjects one year after surgery. We also found NHB subjects had significantly lower baseline triglycerides compared to Hispanics and NHW subjects. This is consistent with other adult<sup>[21-23]</sup> and adolescent studies which found lower triglycerides levels among NHB subjects compared to Hispanics and NHW subjects<sup>[24-26]</sup>.

In conclusion, morbidly obese women with PCOS who have undergone Roux-en-Y gastric bypass surgery show improvement in cardiometabolic disease risk at one year after the procedure. All women demonstrated a decrease in cardiometabolic disease risk factors including BMI, HgA1c, ALT, TC, LDL and TG. However, only Hispanic women showed a significant decreasing HgA1c and ALT, TC, LDL and TG one year after surgery. These ethnic group differences suggest there may be more comprehensive benefits in terms of cardiometabolic disease risk reduction after bariatric surgery in Hispanic women with PCOS *vs* other ethnic groups.

## ACKNOWLEDGMENTS

We are grateful to all the bariatric patients who have agreed to share their data for the current analyses.

## COMMENTS

### Background

It is widely reported that women with polycystic ovarian syndrome (PCOS) have a higher prevalence of cardiometabolic disease risk factors and overt disease compared to non-PCOS women. However, it is less clear how effective bariatric surgery is on the resolution of pre-diabetes, hypertension and dyslipidemia in affected women with PCOS following bariatric surgery, and in multiethnic women in particular.

### Research frontiers

This analysis shows that Roux-en-Y gastric bypass surgery results in a significant decrease in body mass index (BMI) among Hispanic, non-Hispanic black (NHB) and white (NHW) with a history or diagnosis of PCOS. Several ethnic group differences in pre-post change in individual cardiometabolic disease risk factors were found. Specifically, while all ethnic groups showed improvement in hemoglobin A1c (HbA1c) and alanine aminotransferase (ALT), the decrease was significant only in Hispanic subjects. Additionally, all ethnic groups showed improvement in total cholesterol (TC), low-density lipoproteins (LDL) and triglyceride (TG) one year after surgery. However, improvement in TC and TG were only significant for Hispanics and NHB subjects. Decrease in LDL was only significant in Hispanic and NHW subjects.

### Innovations and breakthroughs

This is one of the first studies to demonstrate improvement in cardiometabolic health at one year after the procedure in a large multiethnic sample of morbidly obese women with PCOS. All women demonstrated a decrease in cardiometabolic disease risk factors including BMI, HbA1c, ALT, TC, LDL and TG. However, only Hispanic women showed a significant decreasing HgA1c and ALT, TC, LDL and TG one year after surgery. These ethnic group differences suggest there may be more comprehensive benefits in terms of cardiometabolic disease risk reduction after bariatric surgery in Hispanic women with PCOS *vs* other ethnic groups.

### Applications

Post-surgery, Hispanic women with PCOS had a significant decrease ALT, whereas the decrease in ALT in NHW and NHB subjects was not significant. This decreased in ALT in the Hispanic population may indicate improvement of fatty infiltration after surgery and therefore, weight loss in obese Hispanic women with PCOS may have a greater impact on hepatic steatosis compared to NHW and NHB subjects. This finding suggests multiple improvements post-bariatric surgery among Hispanic women in particular not previously reported.

### Terminology

Obesity, insulin resistance, metabolic syndrome, diabetes, dyslipidemia and nonalcoholic fatty liver disease are commonly seen in women with PCOS. Limited studies have shown that weight loss after bariatric surgery in women with PCOS results in improvement or elimination of the symptoms of PCOS and associated cardiometabolic risk factors. For example, women with PCOS following surgery have demonstrated normalization of menses, hirsutism score, serum androgens, and insulin sensitivity.

### Peer review

The paper may add some new knowledge to the literature.

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## Caribbean maitotoxin elevates $[Ca^{2+}]_i$ and activates non-selective cation channels in HIT-T15 cells

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### Abstract

**AIM:** To investigate the cytotoxic mechanism of caribbean maitotoxin (MTX-C) in mammalian cells.

**METHODS:** We used whole-cell patch-clamp techniques and fluorescence calcium imaging to determine the cellular toxic mechanisms of MTX-C in insulin secreting HIT-T15 cells, which is a system where the effects of MTX have been observed. HIT-T15 cells stably express L-type calcium current, making it a suitable model for this study. Using the fluorescence calcium indicator Indo-1 AM, we found that there is a profound increase in HIT-T15 intracellular free calcium 3 min after application of 200 nmol/L MTX-C.

**RESULTS:** About 3 min after perfusion of MTX-C, a gradual increase in free calcium concentration was observed. This elevation was sustained throughout the entire recording period. Application of MTX-C did not elicit the L-type calcium current, but large cationic

currents appeared after applying MTX-C to the extracellular solution. The current-voltage relationship of the cation current is approximately linear within the voltage range from -60 to 50 mV, but flattened at voltages at -80 and -100 mV. These results indicate that MTX-C induces a non-voltage activated, inward current under normal physiological conditions, which by itself or through a secondary mechanism results in a large amount of cationic influx. The biophysical mechanism of MTX-C is different to its isoform, pacific maitotoxin (MTX-P), when the extracellular calcium is removed.

**CONCLUSION:** We conclude that MTX-C causes the opening of non-selective, non-voltage-activated ion channels, which elevates level of intracellular calcium concentration and leads to cellular toxicities.

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**Key words:** Maitotoxin; Calcium fluorescence; High voltage gated  $Ca^{2+}$  channels; Whole cell patch clamp; Insulin secreting cells

**Core tip:** The toxicity of maitotoxin is estimated to affect over 50000 people annually. Baracuda, snapper, grouper, jacks, and moray eel are particularly notorious for their potential to carry high toxin load. The symptoms of the toxicity include numbness of the perioral area and extremities, reversal of temperature sensation, muscle and joint aches, headache, itching tachycardia, hypertension, blurred vision, and paralysis. Our study aims to elucidate the cellular toxic mechanism of caribbean maitotoxin in mammalian cells. We conclude that it causes opening of non-selective, non-voltage activated ion channels, which elevates level of intracellular calcium concentration and leads to cellular toxicities.

Lu XZ, Deckey R, Jiao GL, Ren HF, Li M. Caribbean maitotoxin elevates  $[Ca^{2+}]_i$  and activates non-selective cation channels in HIT-T15 cells. *World J Diabetes* 2013; 4(3): 70-75 Available

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DOI: <http://dx.doi.org/10.4239/wjd.v4.i3.70>

## INTRODUCTION

Ciguatera fish poisoning is caused by ladder-like polyether toxins<sup>[1]</sup>. Ciguatera occurs circumglobally in tropical coral reef regions, and results from the consumption of fish that have accumulated toxin through the food web<sup>[2-4]</sup>. It is estimated to affect over 50000 people annually, and is no longer a disease limited to the tropics, due both to travel to the tropics and to shipping of tropical fish species to markets elsewhere in the world<sup>[5]</sup>. Large carnivorous fishes associated with coral reefs are the most frequent source of ciguatera<sup>[6]</sup>. Baracuda, snapper, grouper, jacks, and moray eel are particularly notorious for their potential to carry high toxin load<sup>[7]</sup>. The symptoms of ciguatera comprise early onset (2-6 h) gastrointestinal disturbance, including nausea, vomiting, and diarrhea, and may be followed by a variety of later onset (18 h) neurological sequelae, including numbness of the perioral area and extremities, reversal of temperature sensation, muscle and joint aches, headache, itching tachycardia, hypertension, blurred vision, and paralysis<sup>[7-9]</sup>. Ciguatera symptoms in the Caribbean differ somewhat from those in the Pacific in that gastrointestinal symptoms are dominant in Caribbean cases, while in Pacific cases neurological symptoms tend to dominate<sup>[10]</sup>.

Maitotoxin (MTX) is one of toxins implicated in ciguatera. This toxin is the most potent marine toxin known today, with a lethal dose of 0.17 ug/kg<sup>[11]</sup>. MTX elicits calcium influx in all cells and tissues tested<sup>[12,13]</sup>. This calcium influx however, is not mediated by the MTX itself but by activating existing non-selective cation ion channels<sup>[14-16]</sup>. MTX induced calcium influx is observed in pancreatic  $\beta$ -cells<sup>[17-19]</sup>. In  $\beta$ -cells, MTX-induced nonselective cation current is indistinguishable from the insulin stimulating hormone glucagon-like peptide-1 and PACAP-activated current<sup>[20]</sup>. It was reported that the MTX-induced calcium current is dependent on extracellular calcium<sup>[21]</sup>.

We have isolated a novel Caribbean isoform of MTX (MTX-C) from tropical fish. In the present study, we used whole-cell patch-clamp techniques and fluorescence calcium imaging to determine the cytotoxic mechanisms of MTX-C in a hamster pancreatic islet cell line, HIT-T15, which stably expresses L-type calcium currents and thus is a suitable model<sup>[22]</sup>. We also used a pacific maitotoxin (MTX-P) to perform parallel comparisons for all experiments.

## MATERIALS AND METHODS

### Materials

The Caribbean and Pacific maitotoxins were gifts from the Food and Drug Administration of the United States, Mobile, AL.

### Cell culture

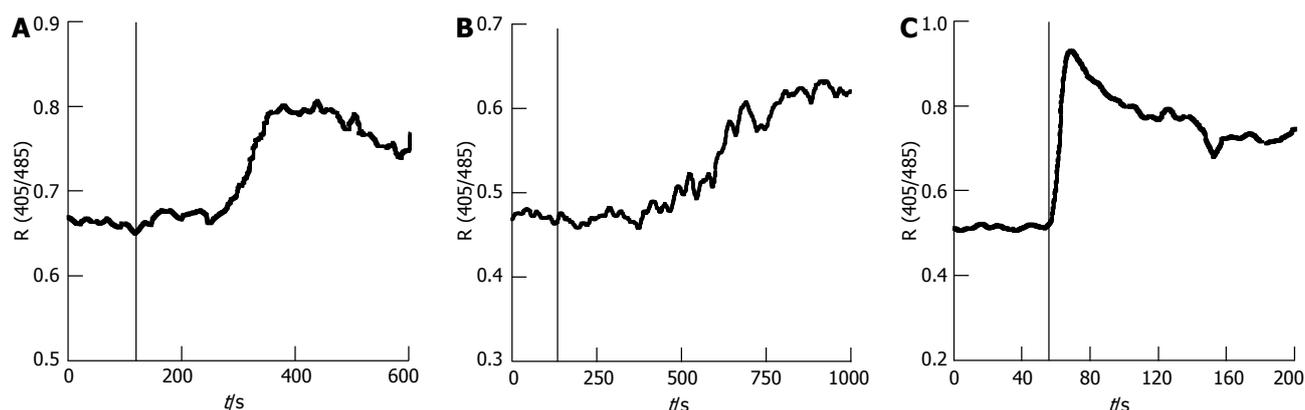
Transformed hamster pancreatic islet cells HIT-T15 (American Type Culture Collection CRL-1777) were routinely cultured in Dulbecco's modified Eagle's medium (25 mmol/L glucose) supplemented with 10% fetal bovine serum (Gibco). For patch clamp recording in the whole-cell and cell-attached configurations, the cells were grown to 30%-80% confluence in 35-mm dishes (Corning). All culture media contained penicillin-G (25 U/mL) and streptomycin (25 mg/mL). Cultures were maintained at 37 °C in 5% CO<sub>2</sub> atmosphere incubator.

### Electrical recording

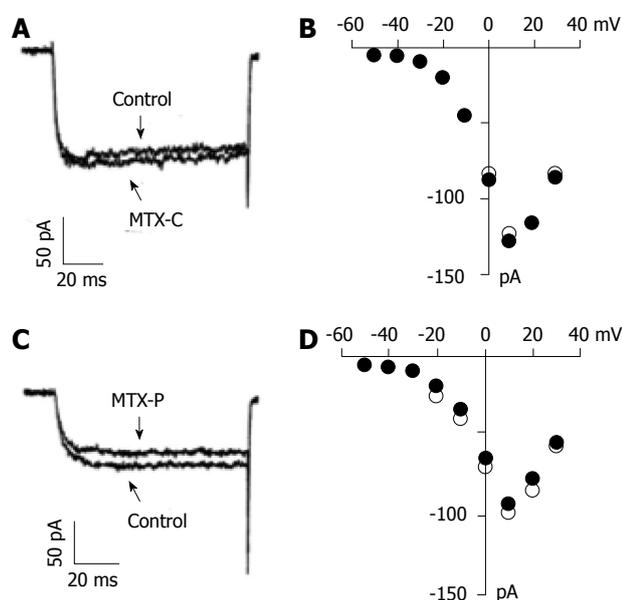
Whole-cell patch clamp recording followed standard techniques. Calcium currents were recorded with an EPC-9 patch clamp amplifier (HEKA Electronics, Lambrecht, Germany). All data were analyzed with PULSE/PULSFIT acquisition and analysis software (HEKA Electronics). The filter frequency was 2.8 kHz and sample frequency was 5 kHz. Patch pipettes were pulled by a two-stage puller (PC-10; Narishige, Greenvale, NY), and heat-polished with a microforge (MF-200; World Precision Instruments, Sarasota, FL) before use. For high voltage gated calcium current measurement, the extracellular solution contained (in mmol/L) 90 TEA-Cl, 40 BaCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPES and 40 sucrose, with pH adjusted to 7.4. The pipette solution contained (in mmol/L) 130 TEA-Cl, 20 EGTA, with pH adjusted to 7.3. For non-selective cation current recording, the extracellular solution contained (in mmol/L): 120 NaCl, 20 TEA-Cl, 5.6 KCl, 2 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 HEPES, with pH adjusted to 7.4; the pipette solution contained (in mmol/L): 130 CsCl, 10 NaCl, 5 Cs-EGTA, 2 MgCl<sub>2</sub>, 5 HEPES, with pH adjusted to 7.4. For cation substitution experiments, bath solutions were the same as the pipette solution except CsCl (120 mmol/L) was replaced by the same amount of KCl or NaCl, accordingly.

### Intracellular calcium fluorescent imaging

Intracellular free calcium concentration was measured in HIT-15 cells using Indo-1 fluorescence imaging. Cells were loaded with 2.5 mmol/L Indo-1 for 30 min. The cells were incubated at 37 °C for 45 min to allow for de-esterification. Fluorescent measurement was conducted by using an ACAS 570 confocal laser scanning microscope. The measurement solution contained (in mmol/L) 135 NaCl, 2 CaCl<sub>2</sub>, 5 KCl, 10 HEPES, with pH adjusted to 7.3. The loading solution consisted of measurement solution augmented with indo-1 AM (2.5  $\mu$ mol/L), DMSO (0.4%) and pluronic F-127 (0.1%). The de-esterification washing solution contained (in mmol/L): 82 Na<sub>2</sub>SO<sub>4</sub>, 30 K<sub>2</sub>SO<sub>4</sub>, 5 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 1 mg/mL BSA. Cells were illuminated with monochromatic light (350 nm), and emitted light was detected at wavelengths of 405 and 485 nm using photomultiplier tubes. The signals from the photomultipliers and force transducer were digitized and stored on a dedicated computer. Data were acquired and analyzed with a separate



**Figure 1** Indo-1 calcium fluorescence measurements of caribbean maitotoxin, pacific maitotoxin and 40 mmol/L of KCl in HIT-T15 cells. The vertical axis represents relative free intracellular calcium concentration as estimated by the ratio of fluorescent emissions at 405 and 485, respectively. The vertical bars in the figure indicate the time when the toxins or KCl were perfused.



**Figure 2** Effect of caribbean maitotoxin (A) and pacific maitotoxin (C) on voltage gated calcium channels in HIT-T15 cells. Representative barium current traces recorded at 10 mV when held at -70 mV in patch clamp. Extracellular solution contains 40 mmol/L BaCl<sub>2</sub>. B and D show I-V relationship of voltage gated calcium currents elicited by caribbean maitotoxin (B) and pacific maitotoxin (D), respectively. Open and solid circles represent values of current before and after application of maitotoxins, respectively.

and after the application of 200 nmol/L MTX-C. About 3 min after perfusion of MTX-C, a gradual increase in free calcium concentration was observed. This elevation was sustained throughout the entire recording period. Similar results were obtained from a MTX-P (10 μmol/L) perfusion experiment, as shown in Figure 1B. The increase of free intracellular calcium started after a delay, and then increased to a plateau over 4-5 min. The pattern of maitotoxin-induced intracellular calcium elevation is noticeably different than the calcium elevation caused by opening of voltage-gated calcium channels where a sharp peak of calcium is followed by a quick decay, as was observed in the same type of cells (Figure 1C).

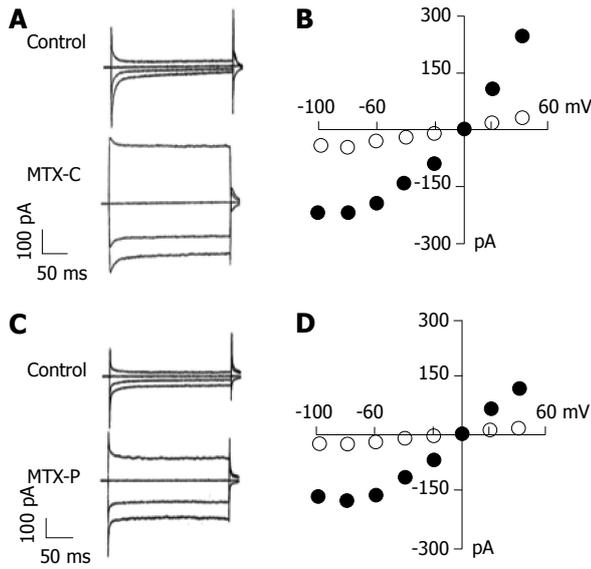
Although the calcium fluorescence experiments suggest that MTX-C might not activate voltage gated calcium channels directly, it is important to determine whether MTX-C alters the kinetics of voltage gated calcium channels. To address this question, whole cell patch clamp experiments were employed to record voltage calcium channel currents and analyze the I-V relationship under conditions with or without the presence of MTX-C or MTX-P. Figure 2A and C show barium current traces measured at 10 mV when held at -70 mV. Figure 2B and D show the I-V plots of the channels before and after adding MTX-C and MTX-P, respectively. These results show that the currents measured in these experiments are generated from the activation of high voltage gated calcium channels. Neither MTX-C nor MTX-P has significant effect on the current amplitude or the voltage dependence of these channels.

Since voltage gated calcium channels are not the primary targets of MTX-C, could its effect of intracellular calcium elevation result from the activation of non-voltage activated cation channels? Experiments were performed to delineate the effect of MTX-C on non-voltage activated cation currents, which were measured using extracellular sodium and intracellular cesium solutions. In these whole cell patch clamp experiments, the membrane potential was held at 0 mV, and the test pulses were from -100 to 50 mV in increments of 10 mV. As shown in Fig-

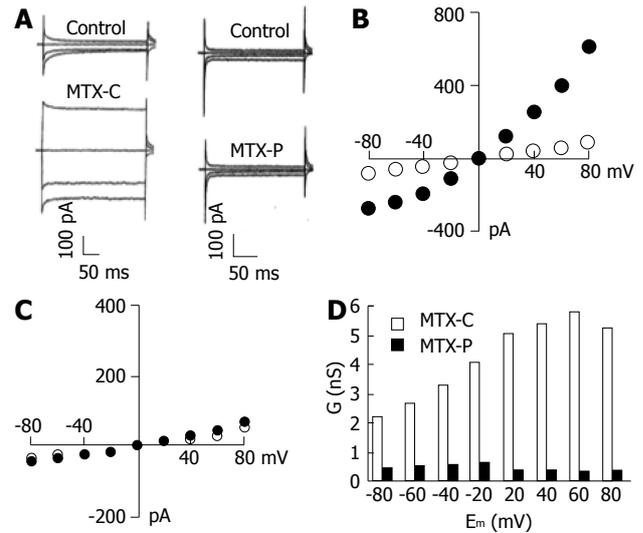
data analysis work station.

## RESULTS

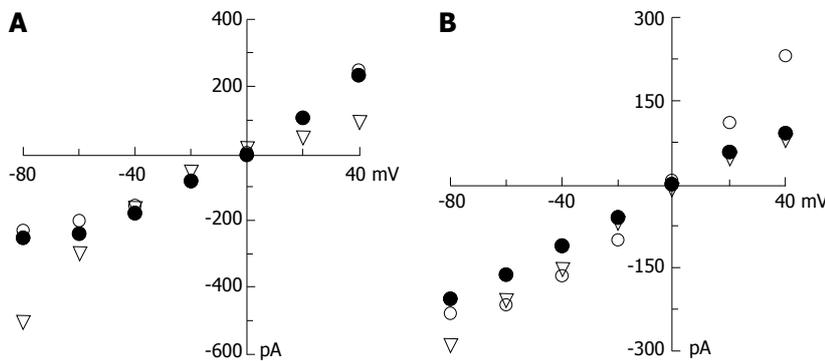
It is known that MTX-P's cytotoxicity is related to disturbing of intracellular calcium homeostasis. In order to determine the effect of MTX-C on intracellular calcium regulation, experiments using the fluorescent dye indo-1 to directly measure the intracellular free calcium concentration were performed in insulin secreting HIT-T15 cells. Both MTX-C and MTX-P induce a sustained elevation in intracellular free calcium concentration. Figure 1A shows the intracellular free calcium concentration before



**Figure 3** Effect of caribbean maitotoxin and pacific maitotoxin on non-voltage activated cation currents in HIT-T15 cells. Representative traces of cation current elicited recorded at -80, -40, 0 and 40 mV before and after adding caribbean maitotoxin (MTX-C) (A) or pacific maitotoxin (MTX-P) (C). The holding potential was at 0 mV. I - V relationships of MTX-C- and MTX-P-elicited currents are shown in B and D, respectively. The solid circles represent current amplitudes measured after MTX-C (B) or MTX-P (D) administration. The open circles represent the current recorded under the control condition.



**Figure 4** Non-voltage activated cation current induced by caribbean maitotoxin or pacific maitotoxin in the calcium free extracellular solution. A: Current traces measured before and after caribbean maitotoxin (MTX-C) or pacific maitotoxin (MTX-P) administration at -80, -40, 0 and 40 mV when held at 0 mV; B and C: I - V relationships of MTX-C (B) and MTX-P (C) elicited currents. The solid circles represent current amplitudes measured after MTX-C (B) or MTX-P (C) administration. The open circles represent the current recorded under the control condition; D: Current conductance measured at different test potentials in cells treated with MTX-C or MTX-P.



**Figure 5** Current and voltage (I - V) relationships of caribbean maitotoxin (A) and pacific maitotoxin (B) opened cation currents. The pipette solution contains 130 mmol/L CsCl; the bath solutions contain 120 mmol/L of CsCl (open circle), NaCl (solid circle) or KCl (triangle).

ure 3, both MTX-C and MTX-P caused profound cation currents, which are approximately linear within the voltage range from -60 to 50 mV, but reduced or flattened at voltages at -80 and -100 mV (Figure 3B and D). Since the electrical driving force for calcium ions are higher at these voltages, this rectification may due to a partial closure of non-voltage gated calcium channels.

Further experiments revealed that these profound non-voltage activated currents induced by MTX-C and MTX-P had a different characterization in extracellular calcium dependency. Under the condition in which EGTA was used to chelate the free extracellular calcium, MTX-P failed to elicit non-voltage activated cation current, as shown in Figure 4A and C. In contrast, this current was continually detected in MTX-C treated cells bathed in the calcium free extracellular solution (Figure 4A and B). However, the inward current conductance was non-linear at voltages lower than 0 mV, as shown in Figure 4D. The discrepancy in calcium-dependent activation of non-

voltage activated current between MTX-C and MTX-P indicates that the toxicological mechanisms in activating non-voltage activated cation currents by the two isoforms of maitotoxin have small but distinct differences.

The selectivity of MTX-C and MTX-P sensitive channels for other cations were also examined. The I - V relationship analyses were performed under conditions of symmetrical CsCl in the pipette and bath solutions, or the bath cesium was replaced by potassium or sodium ions. Since the reversal potentials across at 0 mV for both toxin-elicited currents in all cation solutions, the selectivity is the same for Na<sup>+</sup>, Cs<sup>+</sup> and K<sup>+</sup>. These results suggest that maitotoxins elicit a non-selective, non-voltage activated current. However, for MTX-C, while the I - V relationships in extracellular CsCl were similar to that in NaCl as shown above (Figure 3B), it was very different when the extracellular solution contained KCl, where a clear inward rectification was observed (Figure 5A). In MTX-P sensitive channel experiments, unlike in the NaCl extracellular

solution, this channel showed non-linear reduction at very negative test potential (Figure 3D). The I-V relationships were linear in all voltages between 100 to 50 mV when the bath solutions contain CsCl or KCl (Figure 5B). These results suggest that MTX-C and MTX-P may act on different channel proteins or act on different parts of the same channel protein.

## DISCUSSION

In conclusion, MTX-C causes an opening of non-selective, non-voltage activated ion channels, which permits or elicits further abnormal calcium influx. The elevated level of intracellular calcium concentration resulting from this calcium influx may lead to cellular toxicities.

The toxic potency of MTX-P exceeds that of ciguatera toxins (LD50 0.05/kg *ip* in mice). Its biological activity is strictly calcium dependent and causes both membrane depolarization and calcium influx in many different cell types. It was originally believed to be an activator of voltage dependent calcium channels<sup>[23]</sup>. However, voltage dependent calcium channel antagonists can block MTX-P-stimulated calcium influx, but not MTX-P-induced membrane depolarization<sup>[24]</sup>. The results in the present study show that the opening of non-selective cation channels will result in a net positive ion influx due to the higher electrochemical driving force for sodium ions than for potassium ions. Such a net sodium ion influx can depolarize cell membrane potential and caused activation of voltage gated calcium channels in HIT-T15 cells. This depolarization is a gradual process, which could explain why there was a time delay in the intracellular calcium experiments (Figure 1A and B). MTX-C-induced membrane depolarization also likely causes anomalies of cellular functions and possibly reduces chances of cell survival.

Although the structure of MTX-P has been described previously<sup>[25,26]</sup>, the mechanism of calcium dependence in MTX-P-induced current activation is unclear. It is possible that extracellular calcium creates a significant surface potential that provides a necessary influent to help MTX-P open non-selective cation channels, or it may affect an enzymatic mechanism on the cell surface to achieve the same purpose. The fact that MTX-C could effectively activate these channels regardless of the presence of extracellular calcium indicates that it may be a more potent isoform than MTX-P. However, this likely has little physiological significance since living cells are exposed to an extracellular solution containing calcium ions.

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## COMMENTS

### Background

Ciguatera occurs circumglobally in tropical coral reef regions, and results from the consumption of fish that have accumulated toxin through the food web.

Maitotoxin (MTX) is one of the toxins implicated in ciguatera. MTX is the most potent marine toxin which causes severe illness in gastrointestinal system. MTX presents primarily as an acute neurologic disease manifested by a constellation of gastrointestinal, neurologic and cardiovascular signs and symptoms. Acute fatality, usually due to respiratory failure, circulatory collapse or arrhythmias, ranges from 0.1% to 12% of reported cases. Although the mechanism of pacific isoform of MTX induced cell damage has been studied extensively, the mechanism of caribbean isoform of MTX toxicity is largely unknown.

### Research frontiers

MTX activates Ca<sup>2+</sup> permeable, non-selective cation channels, leading to an increase in levels of cytosolic Ca<sup>2+</sup> ions, which triggers a cell death cascade, resulting in membrane blebbing and eventually cell lysis. MTX is known to activate cytosolic calcium-activated proteases calpain-1 and calpain-2, contributing to necrosis. The molecular characters of MTX activated ion channels, however, remains unknown. This study characterized the electrophysiological differences between currents induced by two isoforms of MTX, provided more insights to the physiological properties of the channel.

### Innovations and breakthroughs

The study shows that pacific and caribbean isoforms of MTX share similar potency in eliciting calcium influx, carried by similar non-selective, non-voltage activated outward-rectified calcium currents. However, the currents induced by two isoforms of MTX had a difference in extracellular calcium dependency: function of pacific MTX is extracellular calcium dependent whereas the caribbean MTX is not. This difference indicates that the toxicological mechanisms is different between the two isoforms of MTX.

### Applications

The conclusion of this study can be applied to further characterize the molecular nature of the MTX activated ion channels and to eventually elucidate the molecular mechanism of MTX toxicity.

### Terminology

Rectified current-voltage relationship: The changes in the size of current is non-linear with the changes of voltage values.

### Peer review

This succinct paper characterizes the effects of caribbean maitotoxin (MTX-C) on Ca<sup>2+</sup> influx in insulin-secreting HIT-T15 cells by means of cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) measurement and whole-cell patch-clamp analysis. [Ca<sup>2+</sup>]<sub>i</sub> measurement reveals that this toxin induces a marked increase in [Ca<sup>2+</sup>]<sub>i</sub>. Whole-cell patch-clamp analysis verifies that MTX-C had no influence on voltage-gated Ca<sup>2+</sup> currents, but evidently activated non-selective cation channels. The data are interesting. They indicate that MTX-C may exert their cellular toxicity by promoting Ca<sup>2+</sup> influx through non-selective cation channels in insulin-secreting HIT-T15 cells.

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## Hemoglobin A1c in early postpartum screening of women with gestational diabetes

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### Abstract

**AIM:** To assess the utility of hemoglobin A1c (HbA1c) in the early postpartum screening of women with gestational diabetes mellitus (GDM).

**METHODS:** Over a 3 years period, HbA1c estimations were undertaken in addition to and simultaneously with the traditional oral glucose tolerance test (OGTT), in 203 women with GDM as a part of early postpartum screening for dysglycaemia, at 6 wk post-partum. World Health Organization criteria was used for diagnosing diabetes: fasting blood glucose (FBG)  $\geq 7.0$  mmol/L

and/or 2-h postprandial blood glucose (PPBG)  $\geq 11.1$  mmol/L and/or HbA1c  $\geq 48$  mmol/mol; and impaired glycaemiastate: impaired fasting glucose 6.1-6.9 mmol/L and/or impaired glucose tolerance 7.8-11.0 mmol/L and/or HbA1c: 42-47 mmol/mol.

**RESULTS:** Mean FBG, 2-h PPBG and HbA1c were  $4.9 \pm 0.7$  mmol/L,  $5.6 \pm 2.0$  mmol/L and  $38 \pm 5$  mmol/mol respectively. FBG, 2-h PPBG and HbA1c detected 6 (3%), 7 (3.5%) and 11 (5.4%) cases of diabetes respectively, and 11 (5.4%), 25 (12.3%) and 23 (11.3%) cases of pre-diabetes state respectively. HbA1c values  $\geq 48$  mmol/mol ( $\geq 6.5\%$ ) showed a diagnostic sensitivity of 71.4% and specificity of 98.5% for diabetes in comparison to OGTT in receiver operating characteristics curve analysis. At HbA1c cut-off 44 mmol/mol, sensitivity and specificity were 100% and 92.3% respectively [area under the curve: 0.98 (95%CI: 0.96-1.00)]. Sensitivity and specificity for detecting high risk "impaired glycaemia" state [HbA1c 42 mmol/mol (6.0%)] were 28% and 80%, respectively.

**CONCLUSION:** HbA1c level  $\geq 48$  mmol/mol ( $\geq 6.5\%$ ) has reasonable sensitivity and high specificity in comparison to OGTT for early postpartum screening of diabetes in GDM. At 6<sup>th</sup> week postpartum screening, if FBG is normal and HbA1c  $< 44$  mmol/mol OGTT is not recommended.

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**Key words:** Postpartum screening; Glycated haemoglobin; Gestational diabetes mellitus; Impaired glycaemia

**Core tip:** Hemoglobin A1c (HbA1c) though accepted as a screening tool for diagnosis of diabetes by professional bodies, its role in early postnatal screening of women with gestational diabetes mellitus is not known, which is explored in this study. Analysing the results of simultaneous oral glucose tolerance test (OGTT) and HbA1c estimations undertaken as a part of postpartum screen-

ing at 6 wk, we note that HbA1c has a high negative predictive value and can help in excluding diabetes (but not impaired glycaemia). We thus propose that HbA1c could potentially be used with fasting blood glucose estimation to avoid OGTT in those women with an HbA1c < 44 mmol/mol.

Katreddy MV, Pappachan JM, Taylor SE, Nevill AM, Indusekhar R, Nayak AU. Hemoglobin A1c in early postpartum screening of women with gestational diabetes. *World J Diabetes* 2013; 4(3): 76-81 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i3/76.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i3.76>

## INTRODUCTION

The global prevalence of diabetes mellitus continues to escalate with the force of an approaching tsunami that affects about 350 million individuals worldwide<sup>[1]</sup>. Increasing prevalence of overweight and obesity in both developed and developing countries are the main factors for the alarming rise in the diabetic epidemic. Alterations in the glucose homeostasis among obese/overweight children and adolescents lead to the peculiar phenomenon of emergence of prediabetes and type 2 diabetes in young adults in the recent years<sup>[2]</sup>. Consequently the prevalence of diabetes among women of child-bearing age is also expected to rise.

Diabetes mellitus is a major cause of perinatal morbidity and mortality, as well as maternal morbidity that complicates about 3%-14% of all pregnancies<sup>[3-7]</sup>. About 85% of diabetes among pregnant women is due to gestational diabetes mellitus (GDM) and the remainder are due to type 2 or type 1 diabetes. It is well recognised that women with GDM have a higher risk of development of impaired glycaemic state or type 2 diabetes over time, with studies suggesting a 17% to 63% risk of developing type 2 diabetes within 5 to 16 years of the index pregnancy<sup>[8]</sup>. The recurrence rate of GDM in a second consecutive pregnancy was found to be about 41% in a recent major study<sup>[9]</sup>. Despite majority of women with GDM becoming normoglycaemic immediately after delivery of the baby, the prevalence of persistent abnormal glucose metabolism (diabetes and impaired glycaemia) in the early postpartum period has been reported to be as high as 46%<sup>[10]</sup>. Identifying these high risk women with persistently impaired glucose regulation and frank diabetes would help in instigating early intervention strategies for better health outcomes. Thus, early postpartum diabetes screening is recommended by professional organizations, for all women with the diagnosis of GDM during pregnancy<sup>[11-15]</sup>.

The American Diabetes Association (ADA) recommends diabetes screening with a 75 g oral glucose tolerance test (OGTT) at 6-12 wk after delivery in women with GDM<sup>[11]</sup> and the World Health Organization (WHO)

recommends similar screening at least 6 wk after delivery<sup>[12]</sup>. A fasting glucose test, instead of the traditional OGTT, at the 6<sup>th</sup> week postpartum visit is recommended by the United Kingdom's National Institute for Health and Clinical Excellence (NICE) for GDM patients as a screening tool<sup>[13]</sup>. Though estimation of glycated hemoglobin A1c (HbA1c) level is accepted as a screening tool for diagnosis of diabetes by WHO and other professional bodies, there is no official recommendation for its use for diabetes screening in the postpartum period.

Because of the ease of estimation of HbA1c in comparison to the cumbersome OGTT, it is being utilized increasingly for screening and diagnosis of diabetes and prediabetes states in the recent years. In this background we conducted a study to explore the utility of HbA1c in the early post-partum screening of women with gestational diabetes in a large university hospital in the United Kingdom.

## MATERIALS AND METHODS

All women who were diagnosed with GDM, managed by diet/lifestyle modifications and/or medical treatment, in the combined antenatal diabetes clinic (with obstetrician, diabetologist, dietician and diabetic specialist nurse) between January 2010 and August 2012, were offered postpartum screening in the 6<sup>th</sup> week postpartum visit. Along with the OGTT, HbA1c estimation was undertaken as a part of the post-partum screening test. All such women were identified and included, and there were no exclusion criteria. These women were given counselling by the diabetic team, during their antenatal follow up, regarding the implications of GDM diagnosis and the need for screening in the post-partum period.

OGTT was performed after a minimum of 8 h of overnight fast, by measuring fasting blood glucose (FBG) and 2-h postprandial blood glucose (PPBG). After taking blood sample for FBG check each participant was given a glucose drink (75 g of D-dextrose powder dissolved in 200 mL of water). Samples for FBG and 2-h PPBG were obtained by taking 2 mL of venous blood in tubes containing sodium fluoride. 3 mL each of venous blood samples were collected in tubes containing EDTA for HbA1c estimation while taking sample for fasting blood glucose. HbA1c was measured using high performance liquid chromatography on a Tosoh G7 analyser (Tosoh Bioscience Ltd., Worcestershire, United Kingdom). The performance scores in the United Kingdom National External Quality Assurance Scheme were: A scores < 100 and B scores < 2%. The between batch coefficient of variation was 1.8% and 1.4% for an HbA1c of 5.7% and 9.5% respectively.

The International Federation of Clinical Chemistry (IFCC) units for HbA1c levels were introduced in the United Kingdom since 1<sup>st</sup> June 2009. Locally, the IFCC reference system was adopted and the dual reporting of HbA1c with IFCC units and the corresponding calculated Diabetes Control and Complications Trial (DCCT)

**Table 1** Categorisation of glycaemia into diabetes, impaired and normal based on the different tests undertaken: fasting blood glucose, oral glucose tolerance test and hemoglobin A1c (*n* = 203) *n* (%)

Category	FBG	OGTT	HbA1c
Diabetes	6 (3.0)	7 (3.5)	11 (5.4)
Impaired	11 (5.4)	25 (12.3)	23 (11.3)
Normal	186 (91.6)	171 (84.2)	169 (83.3)

FBG: Fasting blood glucose; OGTT: Oral glucose tolerance test; HbA1c: Hemoglobin A1c.

value was available during the period and utilised for the analysis of data among the participants. The equation describing the relationship between the IFCC and the DCCT units used was: IFCC-HbA1c (mmol/mol) = [DCCT - HbA1c (%) - 2.15] × 10.929<sup>[16]</sup>.

Data of the test results from participants were collected and they were grouped into categories according to the values as normal, impaired glycaemia or diabetes. FBG values less than 6.1 mmol/L was taken as normal; FBG values between 6.1 mmol/L and 6.9 mmol/L as impaired fasting glucose (IFG); and FBG ≥ 7.0 mmol/L as diabetes. The OGTT results were classified by the WHO criteria: normal glucose tolerance (FBG < 6.0 mmol/L and/or 2-h PPBG < 7.8 mmol/L); impaired glucose tolerance (FBG ≥ 6.1 mmol/L and < 7.0 mmol/L, and/or 2-h PPBG between 7.8 and 11.0 mmol/L); and diabetes (FBG ≥ 7.0 mmol/L and/or 2-h PPBG ≥ 11.1 mmol/L). Glycaemic categorization was also undertaken according to the HbA1c criteria recommended by the WHO in diagnosis of diabetes in the general population: Diabetes (HbA1c ≥ 48 mmol/mol or ≥ 6.5% DCCT) and non-diabetic (HbA1c < 48 mmol/mol or < 6.5%). Those with HbA1c values between 42 mmol/mol and 47 mmol/mol (6.0% and 6.4%) were considered as having an impaired glycaemic state (high risk)<sup>[17]</sup>.

### Statistical analysis

Data was analysed using computer software SPSS Version 19.0 for Windows (SPSS Inc, Chicago, IL, United States). The continuous variables are presented as mean (± 2SD). Differences in the classifications between normal, impaired glycaemia and diabetes using FBG, OGTT and HbA1c were assessed using a non-parametric sign test. Receiver operating characteristics (ROC) curve analysis was used to determine the sensitivity and specificity of HbA1c in comparison with OGTT (as the gold standard) for postpartum screening of diabetes or high risk pre-diabetes state. *P* < 0.05 was considered statistically significant.

## RESULTS

A total of 408 women with gestational diabetes were identified during the study period. However, only 203 women (49.8%) had simultaneous OGTT and HbA1c estimation as a part of the postpartum screening at the 6<sup>th</sup> postpartum week, the cohort which we used in the

**Table 2** Cross tabulation between hemoglobin A1c and oral glucose tolerance test or fasting blood glucose in categorisation of glycaemic status *n* (%)

Glycaemic status		HbA1c		
		Normal	Impaired glycaemia	Diabetes
OGTT	Normal	149 (73.4)	17 (8.4)	5 (2.5)
	Impaired glycaemia	20 (9.9)	4 (2.0)	1 (0.5)
	Diabetes	0 (0)	2 (1.0)	5 (2.5)
FBG	Normal	162 (79.8)	19 (9.4)	5 (2.5)
	Impaired glycaemia	7 (3.4)	3 (1.5)	1 (0.5)
	Diabetes	0 (0)	1 (0.5)	5 (2.5)

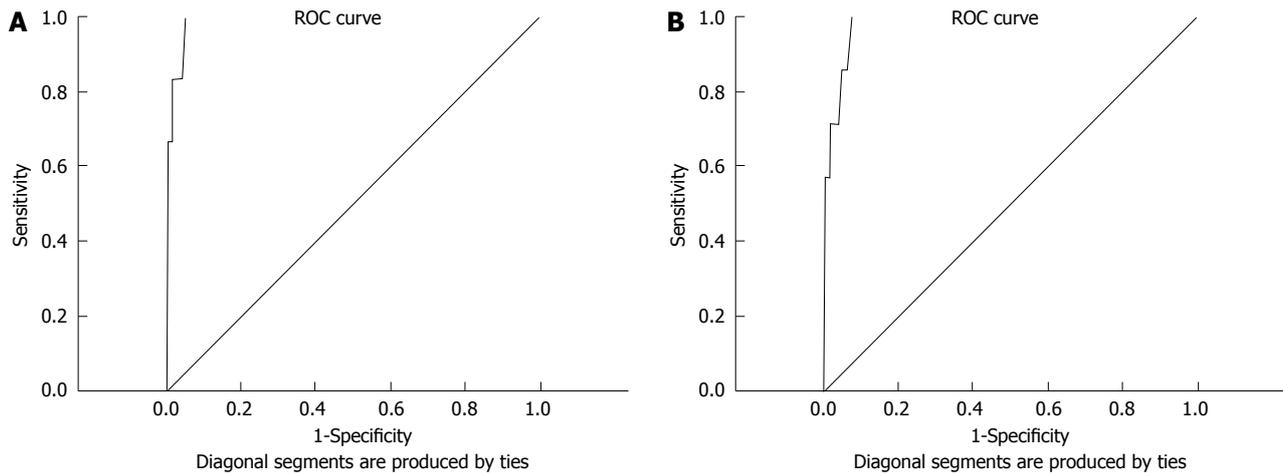
FBG: Fasting blood glucose; OGTT: Oral glucose tolerance test; HbA1c: Hemoglobin A1c.

analysis for this study. Demographic characteristics were as follows: mean age: 29 ± 4.6 years; ethnic origin: 142 were Caucasians (70%) and 61 belonged to other racial groups (Asian: 60, Afro-Caribbean: 2, others: 9); body mass index: 30 ± 6.4 kg/m<sup>2</sup> (Caucasians: 32 ± 5.1 kg/m<sup>2</sup> and Asians 26 ± 4.2 kg/m<sup>2</sup>).

Mean FBG was 4.9 ± 0.7 mmol/L; mean 2-h PPBG 5.6 ± 2.0 mmol/L and HbA1c 38 ± 5 mmol/mol (5.6 ± 0.5%). The correlation for HbA1c *vs* FBG was *r* = 0.42 (*P* < 0.001) and for HbA1c *vs* 2-h PPBG was *r* = 0.42 (*P* < 0.001).

Categorization of the cohort into normal (= 1), impaired glycaemia (= 2) or diabetes (= 3) according to blood glucose and HbA1c criteria (WHO criteria) are shown in Table 1. Using the ADA criteria the prevalence of IGT in OGTT increased from 12.3% to 16.7% without altering the total number diagnosed diabetes<sup>[18]</sup>. The diabetes prevalence using FBG alone and OGTT were 3.0% and 3.5%. Further, OGTT diagnosed a higher proportion with impaired glycaemia compared to FBG (5.4% *vs* 12.3%). Of the 186 women with normal FBG, 15 (8.1%) had IGT on OGTT and none had diabetes based on the 2-h PPBG value on the OGTT. Amongst the 11 women with IFG, 1 had the 2-h PPBG in the diabetes range. Differences in the classifications using FBG and OGTT were assessed using a non-parametric sign test. The misclassifications identified 0 positive, 16 negative differences and 187 ties (*P* < 0.001), suggesting that OGTT is inclined to diagnose a significantly higher proportion of patients with impaired glycaemia and full diabetes compared to FBG.

The categorisation of glycaemia into normal (= 1), impaired glycaemia (= 2) and diabetes (= 3) using HbA1c identified different individuals compared to those identified using the OGTT and FBG (Table 2). HbA1c was in the diabetes range in more women when compared to the OGTT criteria (5.4% *vs* 3.5%, *P* > 0.05). Differences in the classifications using OGTT and HbA1c were also assessed using a non-parametric sign test. The misclassifications identified 22 positive, 23 negative differences and 160 ties (*P* > 0.05), suggesting that HbA1c classified different individuals with normal, impaired glycaemia and diabetes compared to those identified using the OGTT.



**Figure 1** Receiver operating characteristics curve for hemoglobin A1c used for detection of diabetes by glucose criteria. A: Hemoglobin A1c (HbA1c) vs fasting blood glucose [area under the curve 0.99 (95%CI: 0.97-1.00)]; B: HbA1c vs oral glucose tolerance test [area under the curve 0.98 (95%CI: 0.96-1.00)]. ROC: Receiver operating characteristics.

For the diagnosis of diabetes, in the ROC curve (Figure 1), HbA1c values  $\geq 48$  mmol/mol ( $\geq 6.5\%$ ) showed a diagnostic sensitivity 71.4% and specificity 98.5% in comparison to the gold standard OGTT. When the HbA1c cut-off was lowered to 44 mmol/mol the sensitivity 100% and specificity 92.3% [area under the curve 0.98 (95%CI: 0.96-1.00)]. The sensitivity and specificity for detecting high risk impaired glycaemia using cut-off HbA1c 42 mmol/mol (6.0%) were 28% and 80% respectively.

## DISCUSSION

In this study during early post-partum screening at 6 wk for women with GDM all women diagnosed to have diabetes by the OGTT had an abnormality in their fasting blood glucose. Thus, this supports a strategy to undertake FBG as the initial screening test, and a follow-on OGTT only in those with IFG, that would be sufficient to identify all women with diabetes. This is also in line with the current NICE recommendations for the use of FBG as the initial screening test in these women with GDM during early postpartum period. This would be a very cost-effective strategy avoiding OGTT in majority of women with GDM, but is likely to miss a significantly high proportion of women with IGT (8.1% in our study) who potentially may have a higher risk of developing diabetes earlier, than those with normalised postpartum glycaemia, in whom more aggressive interventions have been recommended by some authorities. However, others would argue that gestational diabetes itself is a significant risk for future diabetes, and that having IGT or with normalisation of glycaemia in postpartum period, there should be no practical difference in the degree of intervention and future screening for diabetes.

This study explored the utility of HbA1c in the early postpartum screening of women with gestational diabetes mellitus. Applying the recent WHO HbA1c criteria, the yield of diabetes was significantly higher compared

to the blood glucose (OGTT) criteria (5.4% *vs* 3.5%) and the two tests diagnosed different women with diabetes with concordance only in 2.5%. Previous studies tested the utility of HbA1c in postpartum screening of glucose abnormalities at variable time periods from 6 wk to 36 mo<sup>[10,19-21]</sup>. Our data is unique in that estimation of all the 3 parameters for dysglycaemia (abnormal FBG, OGTT and HbA1c) were done in a single setting during the first postpartum visit at 6 wk. Many patients would have done postpartum screening at other health centres and there remained a variation in the test undertaken in a proportion of women making the response rate low (49.8%).

Use of HbA1c has been recommended as a test to diagnose diabetes by various international organisations<sup>[22-25]</sup>. Advantage of blood sampling in the non-fasting state without the need for OGTT makes HbA1c check as a promising tool for diabetes screening. However, the reliability of HbA1c as a true reflection of glycaemic status at 6 wk post-partum could be affected by several factors including glucose control in the later stages of pregnancy and/or the use or discontinuation of hypoglycaemic therapies peri-partum. HbA1c represents average glucose levels over the last 3 mo, not 6 wk, and therefore HbA1c at 6 wk postpartum partly reflects glucose levels during the pregnancy and hence the concern to use it at 6 wk post-partum as a screening test. The performance of HbA1c is likely better at about 12 wk or 3 mo postpartum. In addition, altered red cell turnover, anaemia of pregnancy and potential factors altering glycation - deglycation rates in the erythrocyte might affect the HbA1c value independent of prevailing glucose control that affect the categorisation of glycaemic status<sup>[26]</sup>. In our study higher proportion of patients were found to have HbA1c in the diabetes range compared to the previous reports, which may be due to the impact of prevailing glucose control just prior to delivery on the HbA1c estimation at 6 wk post-partum<sup>[19,20]</sup>.

Though HbA1c did not appear to help in the diagno-

sis of diabetes in this study, it was noted that HbA1c had a high negative predictive value/sensitivity, with HbA1c cut-off < 44 mmol/mol practically excluding all those with diabetes (but not IGT). Thus HbA1c may be potentially used with FBG estimation to avoid OGTT in those women with an HbA1c < 44 mmol/mol. Further studies with larger cohort may be required to confirm this and prior to recommending HbA1c on its own as the test for early post-partum screening for women with gestational diabetes mellitus.

## COMMENTS

### Background

Hemoglobin A1c (HbA1c) has been accepted as a screening tool for diabetes mellitus by various professional bodies in the recent years. However, there is no data available on the efficacy of HbA1c in comparison to oral glucose tolerance test (OGTT) for screening diabetes and impaired glycaemia at the 6<sup>th</sup> postpartum week in patients with gestational diabetes mellitus (GDM).

### Research frontiers

OGTT, the gold standard test for postpartum screening of GDM cases, is cumbersome and the patients need to fast overnight, whereas HbA1c estimation needs only random blood testing. Limited data on postpartum screening with HbA1c at different timescales after childbirth showed variable results in terms of the sensitivity and specificity. It remains unclear if HbA1c estimation can be used as a screening tool for diabetes and impaired glycaemia at the 6<sup>th</sup> week postpartum clinic visit.

### Innovations and breakthroughs

This study demonstrated that HbA1c has reasonable sensitivity and high specificity in comparison to OGTT for postpartum screening of diabetes in patients with GDM.

### Applications

Results of the study shows that GDM patients with HbA1c values less than 44 mmol/mol and normal fasting blood glucose levels may not need the cumbersome OGTT to exclude diabetes mellitus.

### Peer review

HbA1c levels reflect the average glycaemic burden over 3-mo period and estimation of HbA1c at the 6<sup>th</sup> postpartum week may overestimate prevalence of diabetes in those with high glucose levels towards term of pregnancy and therefore may limit the diagnostic utility in screening patients with GDM. However, the study demonstrated reasonable sensitivity, high specificity of HbA1c as a postpartum screening tool.

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## Atherosclerotic cardiovascular disease among patients with type 2 diabetes in Basrah

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### Abstract

**AIM:** To determine the frequency of atherosclerotic cardiovascular disease and its risk factors among patients with type 2 diabetes in Basrah, Iraq.

**METHODS:** Participants in this cross-sectional study were patients who had type 2 diabetes for at least 1 year, presenting at the Al-Faiha Diabetes Endocrine and Metabolism Center in Basrah (Southern Iraq) over the period from January to December 2008.

**RESULTS:** The series included 1079 patients (58.8% men), of whom 25.0% were smokers. The prevalence of symptomatic cardiovascular disease and hypertension was 16.0%, and 44.3% respectively. Those who were overweight or obese constituted 70.5%. Insulin was used in only 26.9% despite 56.1% having had diabetes for 6 years and more. The mean glycosylated hemoglobin (HbA1c) was  $9.46\% \pm 2.0\%$  and only 5.5% achieved the target of HbA1c of  $< 7\%$ . We had 68.7% of patients with total cholesterol of  $\geq 200$  mg/dL, 21.5% with high density lipoprotein cholesterol of  $< 40$  mg/dL, 84.1% with low density lipoprotein cholesterol of  $\geq 100$  mg/dL and 71.6% with triglyceride of  $\geq 150$  mg/dL.

**CONCLUSION:** Among adults with type 2 diabetes mellitus, there was increased frequency of cardiovascular disease and its modifiable risk factors. This finding necessitated urgent work to modify these risk factors in a population based setting.

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**Key words:** Cardiovascular risk factors; Diabetes mellitus; Type 2 diabetes mellitus; Cardiovascular disease

**Core tip:** Atherosclerotic cardiovascular complications are mainly responsible for the high morbidity and mortality in people with diabetes. We studied 1079 patients with type 2 diabetes mellitus to determine the frequency of atherosclerotic cardiovascular disease and its risk factors. The main finding of this study was an increased frequency of cardiovascular disease and its modifiable risk factors among patients with type 2 diabetes in Basrah, Iraq.

Mansour AA, Ajeel NAH. Atherosclerotic cardiovascular disease among patients with type 2 diabetes in Basrah. *World J Diabetes* 2013; 4(3): 82-87 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i3/82.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i3.82>

### INTRODUCTION

In 2004, the World Health Organization projected that by 2030, the global prevalence of diabetes would double, to affect an estimated 366 million individuals, representing 4.4% of the world population<sup>[1]</sup>.

Atherosclerotic cardiovascular complications are mainly responsible for the high morbidity and mortality in people with diabetes<sup>[2-4]</sup>. Cardiovascular disease (CVD), which includes coronary heart disease (CHD), and stroke, accounts for up to 80% of all deaths among patients with

diabetes<sup>[5]</sup>, compared with one-third of all deaths in the general population<sup>[6]</sup>. This CVD rate in people with type 2 diabetes also varies according to ethnicity<sup>[7]</sup>.

Atherosclerosis in diabetes is more precocious, aggressive and multi-focal than in the non-diabetic population<sup>[8]</sup>. That is why it is recommended to decrease low density lipoprotein cholesterol (LDL-C) < 100 mg/dL (< 70 mg/dL in people with overt CHD with diabetes)<sup>[9]</sup>. From a cardiovascular point of view, it has been suggested that diabetic patients should be considered as about 15 years older than the general population<sup>[10]</sup>. The chance of cardiovascular events in diabetic patients is as high as in patients with a previous history of myocardial infarction but without diabetes<sup>[11]</sup>. Risk factors for CVD in people with diabetes include age, duration of diabetes, smoking, abnormal lipid profiles, raised blood pressure and albuminuria<sup>[12,13]</sup>.

The aim of this study was to determine the frequency of atherosclerotic CVD and its risk factors among patients with type 2 diabetes in Basrah, Iraq.

## MATERIALS AND METHODS

Participants in this cross-sectional study were patients who had type 2 diabetes for at least 1 year, presenting at the Al-Faiha Diabetes, Endocrine and Metabolism Center in Basrah (Southern Iraq) over the period from January to December 2008 and who had not received lipid lowering agents prior to enrollment. This center received patients from all over Basrah and it is the only qualified center treating diabetes in Southern Iraq. Participants gave written consents and the ethical committee of Basrah College of Medicine approved the study.

The definition of CVD included CHD and cerebrovascular accidents (CVA; stroke). CHD diagnosis was based on a history of admission to CCU with elevated cardiac biomarkers, electrocardiographic evidence of ST elevation, Q wave myocardial infarction or left bundle branch block, echocardiographic segmental wall motion abnormalities, abnormal angio-cardiography, percutaneous coronary intervention or coronary artery bypass surgery. The CVA was diagnosed on the basis of sudden neurological deficit that lasted for 24 h, with or without neuroimaging changes.

### Variables risk factors for CVD

Potential risk factors for CVD included age, gender, smoking status, waist circumference (WC), body mass index (BMI), hypertension, duration of diabetes, glycated hemoglobin (HbA1c), insulin use, serum total cholesterol and high density lipoprotein cholesterol (HDL-C), triglycerides (TG), and LDL-C. Current smoker was defined as any person who had smoked cigarettes in the preceding three months, regardless of the number.

Hypertension was defined as systolic blood pressure of 140 mmHg or higher, diastolic blood pressure of 90 mmHg or higher, use of antihypertensive agents or a combination of these. Blood pressure was measured in a

seated position after 5 min at rest, three times. The average of the second and third readings was defined as the subject's blood pressure.

Diabetes mellitus was defined as plasma glucose level being 200 mg/dL or higher independent of fasting time, fasting plasma glucose of  $\geq 126$  mg/dL on two occasions, use of anti-diabetes agents or a combination of these<sup>[14]</sup>. After failure of oral antihyperglycemic agents failed we started with basal human insulin neutral protamine Hagedorn at bedtime then basal prandial or pre-mixed twice.

### Anthropometric measurements

The waist circumference, was measured in centimeters using a plastic anthropometric tape from the horizontal plane at the umbilical level. Measurement was made with the participant standing erect, abdomen relaxed, arms at the side and feet together with weight equally divided over both legs. Participants were told to breathe normally and to breathe out gently at the time of measurement and not to hold in their abdomen or hold their breath. BMI was calculated as body weight in kilograms divided by the squared value of body height in meters ( $\text{kg}/\text{m}^2$ ).

### Biochemical analysis

Fasting serum samples were analyzed in the same local laboratory. Blood (10 mL) was drawn into a tube after the subject had fasted for at least 10 h. Serum total cholesterol, HDL-C, and TG were measured by the enzymatic method. LDL-C concentrations were calculated using the Friedewald formula. Risk factors in the lipoprotein profile of participants would be a total cholesterol of equal or more than 200 mg/dL, LDL-C equal or more than 100 mg/dL, HDL-C less than 40 mg/dL and TG level equal or more than 150 mg/dL<sup>[14,15]</sup>. HbA1c was measured using a fully automated glycohemoglobin analyzer which uses high performance liquid chromatography.

### Statistical analysis

Data analysis was performed using the Statistical Package for Social Sciences (SPSS) version 15 (SPSS Inc, Chicago, Illinois, United States). Characteristics of the sample are reported as mean  $\pm$  SD or frequencies and percentages. Pearson's  $\chi^2$  was used to examine if there is an association between two categorical variables and Student's *t* test were used for continuous variables.

## RESULTS

Table 1 shows the basic socio-demographic and cardiovascular risk factors. The series included 1079 patients (58.8% men, mean age  $56.3 \pm 11.37$  years; age range, 26-94 years) with a mean qualification period of  $6.0 \pm 6.0$  years and 25.0% were current smokers. The mean duration of type 2 diabetes was of  $7.4 \pm 6.5$  years.

The prevalence of symptomatic CVD was 16.0% and hypertension was seen in 44.3%. The mean WC was  $100.2 \pm 12.3$  cm and BMI  $28.0 \pm 5.4$   $\text{kg}/\text{m}^2$ . Those who

**Table 1 Sociodemographic and cardiovascular risk factors of 1079 patients *n* (%)**

Variable		
Sex	Men	634 (58.8)
	Women	445 (41.2)
Age (yr)	mean ± SD	56.3 ± 11.37
	< 40	83 (7.7)
	40-49	220 (20.4)
	50-59	330 (30.6)
	≥ 60	41.3 (41.3)
Education (yr)	mean ± SD	6.0 ± 6.0
	Illiterate	455 (42.2)
	≤ 9	326 (30.2)
	≥ 10	298 (27.6)
Current smoking	Yes	270 (25.0)
	No	809 (75.0)
Duration of diabetes (yr)	mean ± SD	7.4 ± 6.5
	≤ 5	474 (43.9)
	6-10	361 (33.5)
	> 10	244 (22.6)
Known cardiovascular disease	Yes	173 (16.0)
	No	906 (83.9)
Hypertension	Yes	478 (44.3)
	No	601 (55.7)
WC (cm)	mean ± SD	100.2 ± 12.3
BMI	mean ± SD	28.0 ± 5.4
	< 25	318 (29.5)
	25-29	396 (36.7)
	≥ 30	365 (33.8)
Insulin use	Yes	290 (26.9)
	No	789 (73.1)
HbA1c (% total Hb)	mean ± SD	9.46 ± 2.0
	< 7	59 (5.5)
	≥ 7	1020 (94.5)
Total cholesterol (mg/dL)	mean ± SD	206.8 ± 42.6
	< 200	338 (31.3)
	≥ 200	741 (68.7)
HDL cholesterol (mg/dL)	mean ± SD	46.5 ± 13.0
	< 40	232 (21.5)
	≥ 40	847 (78.5)
LDL cholesterol (mg/dL)	mean ± SD	122.6 ± 35.9
	< 100	172 (15.9)
	≥ 100	907 (84.1)
Triglycerides (mg/dL)	mean ± SD	193.5 ± 98.3
	< 150	306 (28.4)
	≥ 150	773 (71.6)

WC: Waist circumference; BMI: Body mass index; HbA1c: Hemoglobin A1c test; HDL: High density lipoprotein; LDL: Low density lipoprotein.

were overweight or obese constituted 70.5%. Insulin was used in 26.9% despite the fact that 56.1% had diabetes for 6 years and more. The mean HbA1c was 9.46 % ± 2.0% and only 5.5% achieved the target of HbA1c of < 7%. We had 68.7% of patients with total cholesterol of ≥ 200 mg/dL, 21.5 % with HDL-C of < 40 mg/dL, 84.1% with LDL-C of ≥ 100 mg/dL and 71.6 % with TG of ≥ 150 mg/dL.

In Table 2, the sociodemographic and cardiovascular risk factors are shown according to gender. There were no differences between the two groups in the age or duration of diabetes. Qualification levels were statistically higher among men, as was the smoking rate ( $P < 0.0001$ ). Among the 25% current smokers, 87.8% were men. Established CVD and hypertension levels were higher in

**Table 2 Sociodemographic and cardiovascular risk factors of 1079 patients according to gender *n* (%)**

Variable		Men	Women	<i>P</i> value
Age (yr)	mean ± SD	56.6 ± 11.5	55.8 ± 11.0	0.276
	< 40	44 (53.0)	39 (47.0)	0.650
	40-49	133 (60.5)	87 (39.5)	
	50-59	191 (57.9)	139 (42.1)	
	≥ 60	266 (59.6)	180 (40.4)	
Education (yr)	mean ± SD	8.2 ± 2.8	3.0 ± 4.7	< 0.0001
	Illiterate	160 (35.2)	295 (64.8)	< 0.0001
	≤ 9	228 (69.9)	98 (30.1)	
	≥ 10	246 (82.6)	52 (17.4)	
Current smoker	Yes	237 (87.8)	33 (12.2)	< 0.0001
Duration of diabetes (yr)	mean ± SD	7.6 ± 6.9	7.1101 ± 6.02	0.149
	≤ 5	267 (56.3)	207 (43.7)	0.314
	6-10	222 (61.5)	139 (38.5)	
	> 10	145 (59.4)	99 (40.6)	
Known cardiovascular disease		125 (72.3)	48 (27.7)	< 0.0001
Hypertension		265 (55.4)	213 (44.6)	0.048
WC (cm)	mean ± SD	100.15 ± 12.45	100.46 ± 12.16	0.681
BMI	mean ± SD	27.7 ± 5.0	28.5 ± 5.9	0.029
	< 25	190 (59.7)	128 (40.3)	0.193
	25-29	243 (61.4)	153 (38.6)	
	≥ 30	201 (55.1)	164 (44.9)	
Insulin use	Yes	129 (44.5)	161 (55.5)	< 0.0001
HbA1c	mean ± SD	9.32 ± 2.0	9.61 ± 2.05	0.100
(% total Hb)	< 7	35 (59.3)	24 (40.7)	0.928
	≥ 7	599 (58.7)	421 (41.3)	
Total cholesterol (mg/dL)	mean ± SD	206.2 ± 42.6	207.7 ± 42.6	0.658
	< 200	221 (65.4)	117 (34.6)	0.003
	≥ 200	413 (55.7)	328 (44.3)	
HDL cholesterol (mg/dL)	mean ± SD	45.85 ± 14.58	47.76 ± 9.84	0.040
	< 40	173 (74.6)	59 (25.4)	< 0.0001
	≥ 40	461 (54.4)	386 (45.6)	
LDL cholesterol (mg/dL)	mean ± SD	122.2 ± 35.5	123.2 ± 36.5	0.723
	< 100	106 (61.6)	66 (38.4)	0.447
	≥ 100	528 (58.2)	379 (41.8)	
Triglycerides (mg/dL)	mean ± SD	201.0 ± 107.1	181.3 ± 80.60	0.004
	< 150	179 (58.5)	127 (41.5)	0.913
	≥ 150	455 (58.9)	318 (41.1)	

WC: Waist circumference; BMI: Body mass index; HbA1c: Hemoglobin A1c test; HDL: High density lipoprotein; LDL: Low density lipoprotein.

men ( $P < 0.0001$  and  $P = 0.048$  respectively). There was no difference in the WC but the BMI was higher among women ( $P = 0.029$ ). Insulin was used more commonly among women ( $P < 0.0001$ ), but no differences were seen in the mean HbA1c. No significant differences were seen between the two groups regarding total cholesterol or LDL-C. The HDL-C was higher among women ( $P = 0.040$ ) while TG was higher among men ( $P = 0.004$ ). Subgroup analysis of the two groups showed total cholesterol ≥ 200 mg/dL and HDL-C < 40 mg/dL was more common among men ( $P = 0.003$  and  $P < 0.0001$  respectively).

The percentage of patients with hypertension, current smoker status and levels of HbA1c, LDL-C, and HDL-C outside the target levels according to the studied variables are summarized in Table 3. Men were more likely to be current smokers and have HDL-C < 40 mg/dL, whereas women were more likely to be hypertensive. There were no differences in risk factors between age groups. Illiterates were more likely to be hypertensive and have HbA1c

**Table 3 Percentages of patients with type 2 diabetes (*n* = 1079) with cardiovascular risk factors outside target range**

Variable (%)	Current smoking	Blood pressure ( $\geq$ 140/90 mmHg)	HbA1c ( $\geq$ 7%)	LDL cholesterol ( $\geq$ 100 mg/dL)	HDL cholesterol ( $<$ 40 mg/dL)
All	25.0	44.3	86.1	84.1	27.6
Gender					
Men	37.4	41.8	84.4	83.3	32.8
Women	7.4	47.9	87.8	85.2	18.7
<i>P</i> value	< 0.0001	0.048	0.267	0.447	< 0.0001
Age (yr)					
< 40	27.7	45.8	87.2	84.3	33.9
40-49	26.8	39.5	87.3	81.4	26.2
50-59	21.2	45.5	84.4	83.9	27.4
$\geq$ 60	26.5	45.5	86.4	85.4	27.3
<i>P</i> value	0.291	0.469	0.905	0.610	0.727
Education (yr)					
Illiterate	19.3	52.3	86.6	87.9	26.1
$\leq$ 9	31.0	35.3	90.7	82.8	27.8
$\geq$ 10	27.2	41.9	79.4	79.5	29.2
<i>P</i> value	0.001	< 0.0001	0.020	0.007	0.693
Duration (yr)					
$\leq$ 5	25.3	28.7	85.4	84.6	24.6
6-10	26.3	58.4	85.2	84.8	28.5
> 10	22.3	55.8	88.6	82.0	31.7
<i>P</i> value	0.545	< 0.0001	0.672	0.596	0.207
BMI (kg/m <sup>2</sup> )					
< 25	25.8	31.1	84.0	84.9	28.4
25-29.9	23.2	47.0	84.6	81.6	26.6
$\geq$ 30	26.3	52.9	89.7	86.0	27.9
<i>P</i> value	0.579	< 0.0001	0.254	0.216	0.873
WC (cm)					
< 80	25.9	15.5	88.6	81.0	23.7
$\geq$ 80	25.0	45.9	85.9	84.2	27.7
<i>P</i> value	0.879	< 0.0001	0.656	0.466	0.585
Insulin use					
Yes	18.3	40.0	92.8	79.7	26.3
No	27.5	45.9	80.7	85.7	28.0
<i>P</i> value	0.002	0.085	< 0.0001	0.019	0.624

WC: Waist circumference; BMI: Body mass index; HbA1c: Hemoglobin A1c test; HDL: High density lipoprotein; LDL: Low density lipoprotein.

> 7%. Those with a duration of diabetes greater than 5 years and those with high BMI and WC  $\geq$  80 cm were more likely to be hypertensive. Insulin was used less in those who were smokers, and had LDL-C  $\geq$  100 mg/dL but was used more in those with HbA1c  $\geq$  7%.

## DISCUSSION

The prevalence of symptomatic CVD in our study was 16.0%; Current smokers were 25.0%; Hypertension was seen in 44.3% and 70.5% were overweight or obese. In Kuwait, 59.3% of patients with type 2 diabetes were hypertensive, 26.3% were overweight, 65.0% were obese, history of CVD was present in 10.0% of patients and 13.3% were smokers<sup>[16]</sup>. Among centers for diabetes, at the University of Jordan, Amman, in patients with type 2 diabetes approximately 70.0% of the patients had hypertension<sup>[17]</sup>.

In a cohort study of diabetic patients in Lebanon, 43.8% patients were obese, 35.0% were current smokers and hypertension was present in 53.2%<sup>[18]</sup>. Among 1846 patients with diabetes in Spain, obesity and high blood pressure were present in > 50.0%, and established CVD in 24.0%<sup>[19]</sup>. Of patients with type 2 diabetes in a district

of Madrid, Spain, only 13.0% had a BMI < 25 kg/m<sup>2</sup><sup>[20]</sup>.

In patients with type 2 diabetes mellitus at primary care settings from the North Catalonia Diabetes Study, CVD prevalence was 22.0%, hypertension 74.5%, smoking 14.9% and obesity 44.9%<sup>[21]</sup>. Overall, 27.0% of diabetic American Indians reported a history of CVD and 57.0% had hypertension<sup>[22]</sup>. Zhang *et al*<sup>[23]</sup> found that in 3469 type 2 diabetic in-patients in China hypertension was seen in 41.8%, CHD in 25.1%, cerebral vascular disease in 17.3%.

In our study the mean HbA1c was 9.46%  $\pm$  2.0% and only 5.5% achieved the target HbA1c of < 7% while in Kuwait it was 16.2%<sup>[16]</sup>, in Spain 41%<sup>[20]</sup>, in Germany 46.6%<sup>[24]</sup> and in Lebanon, 32% of patients with type 2 diabetes had HbA1c of  $\leq$  7%<sup>[18]</sup>. The association between HbA1c and CVD in type 2 diabetes is well known with a relative risk of 1.18 (95%CI: 1.10-1.26) for CVD with a 1% increase in HbA1c<sup>[25]</sup>.

The insulin was used in our study by 26.9 % of patients despite the fact that 56.1 % had diabetes for 6 years and more. In Lebanon, only 20.0% of patients were taking insulin<sup>[18]</sup>.

In this study, majority of our patients had lipid profiles outside of the target range. In one study on lipid

profile in apparently healthy volunteers in Mosul, Iraq, 42% of the study population had TG > 150 mg/dL, 58% had LDL-C > 100 mg/dL, and 50 % had HDL-C < 40 mg/dL<sup>[26]</sup>. There was no difference in the prevalence of elevated LDL-C by the studied variables except for less use of insulin, a finding that was also seen in Kuwaiti patients<sup>[16]</sup>. In Kuwait, 86.2% overall and 63.2% of the patients with type 2 diabetes had LDL-C > 100 mg/dL and HDL-C outside the target levels<sup>[16]</sup>. The North Catalonia Diabetes Study showed dyslipidemia in 77.7%<sup>[21]</sup>. In Jordan, patients with type 2 diabetes had high LDL-C ( $\geq$  100 mg/dL) in 91.5%, low HDL-C in 83.9%, high TG in 83.1% and high total cholesterol in 77.2%<sup>[17]</sup>. In Augsburg Diabetes Family Study, total cholesterol was seen as < 200 mg/dL in 44.1%, and LDL-C was < 100 mg/dL in 16.0% of the participants<sup>[24]</sup>.

In this study the smoking rate among men was higher than in other studies. Established CVD and hypertension were found more in men, while BMI was higher among women. In Kuwait, hypertension was more prevalent among women than men and smoking was only present among men<sup>[16]</sup>. The BMI, and HDL-C were significantly higher in women than men, while TG and total cholesterol was higher among men in Kuwait.

In conclusion, our study demonstrated that among adults with type 2 diabetes mellitus, there was increased frequency of cardiovascular disease and its modifiable risk factors. This finding suggests a need for urgent work to modify these risk factors in the population-based setting.

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## COMMENTS

### Background

Atherosclerotic cardiovascular complications are mainly responsible for the high morbidity and mortality in people with diabetes. Cardiovascular disease (CVD), which includes coronary heart disease, and stroke, accounts for up to 80% of all deaths among patients with diabetes, compared with one-third of all deaths in the general population. The CVD rate in people with type 2 diabetes also varies according to ethnicity.

### Research frontiers

The prevalence of symptomatic CVD was 16.0% and hypertension was seen in 44.3% of patients. Those who were overweight or obese constituted 70.5%. Only 5.5% achieved the target of hypertension, duration of diabetes, and glycosylated hemoglobin (HbA1c) of < 7%. The authors found 68.7% of patients with total cholesterol of  $\geq$  200 mg/dL, 21.5% with high density lipoprotein cholesterol (HDL-C) of < 40 mg/dL, 84.1% with low density lipoprotein cholesterol (LDL-C) of  $\geq$  100 mg/dL and 71.6 % with triglycerides (TG) of  $\geq$  150 mg/dL.

### Innovations and breakthroughs

Potential risk factors for CVD included age, gender, smoking status, waist circumference, body mass index, hypertension, duration of diabetes, HbA1c, insulin use, serum total cholesterol and HDL-C, TG and LDL-C. This study demonstrated that among adults with type 2 diabetes mellitus, there was increased

frequency of cardiovascular disease and its modifiable risk factors.

### Applications

This finding suggests a need for urgent work to modify these risk factors in the population-based setting.

### Peer review

Well written study report provides epidemiological data from a cohort of type 2 diabetes patients in Iraq.

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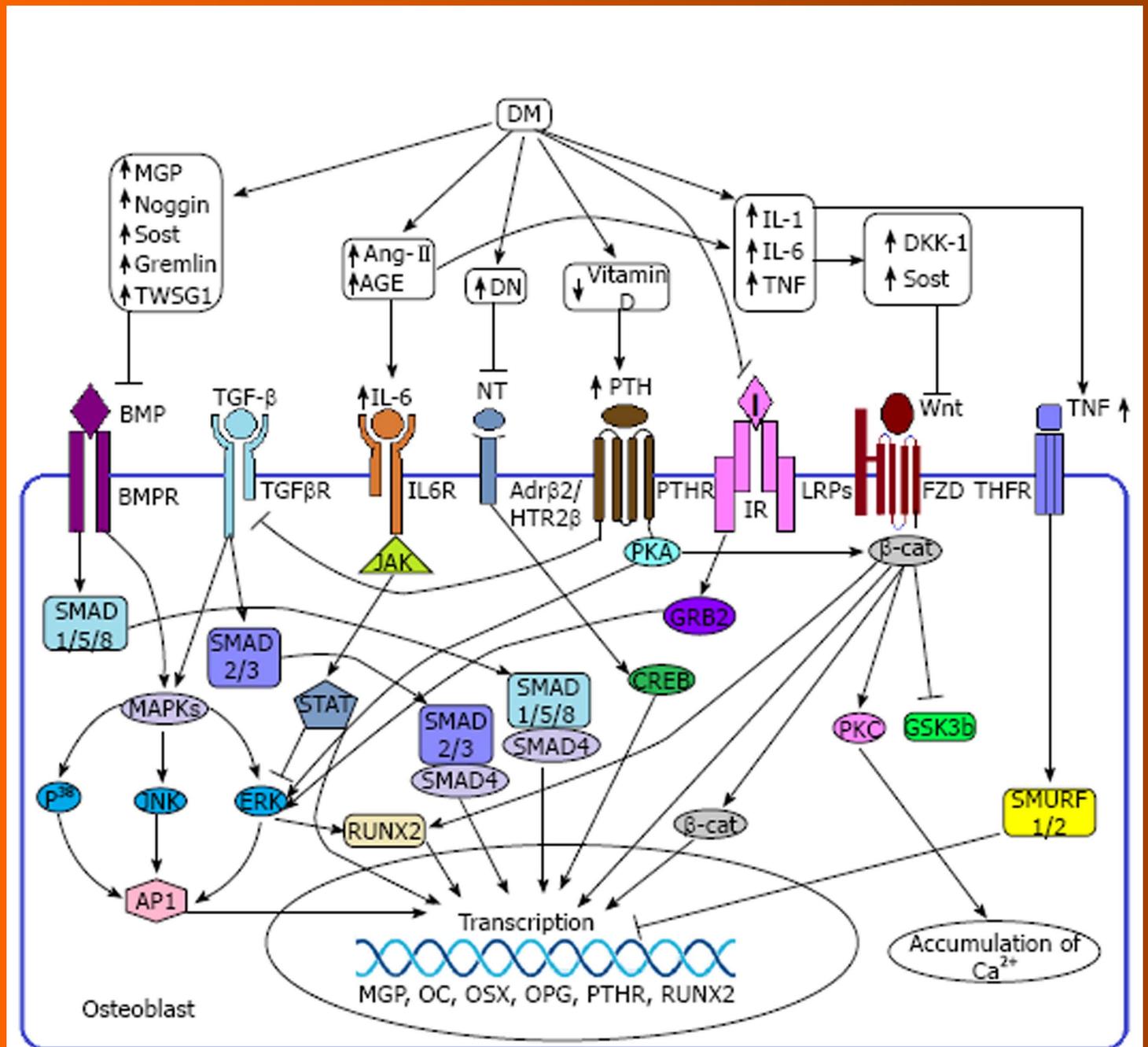
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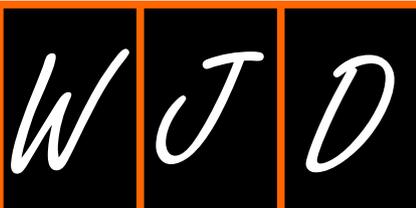
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World J Diabetes 2013 August 15; 4(4): 88-161





<b>FIELD OF VISION</b>	88	Status of autoimmune diabetes 20-year after generation of BDC2.5-TCR transgenic non-obese diabetic mouse <i>Ramirez L, Hamad ARA</i>
<b>REVIEW</b>	92	Trace elements in diabetic cardiomyopathy: An electrophysiological overview <i>Ozturk N, Olgar Y, Ozdemir S</i>
	101	Biomolecular basis of the role of diabetes mellitus in osteoporosis and bone fractures <i>Roy B</i>
<b>MINIREVIEWS</b>	114	Genetics of type 2 diabetes <i>Ali O</i>
	124	Diabetic nephropathy: Treatment with phosphodiesterase type 5 inhibitors <i>Thompson CS</i>
	130	Type 1 diabetes and celiac disease: The effects of gluten free diet on metabolic control <i>Scaramuzza AE, Mantegazza C, Bosetti A, Zuccotti GV</i>
<b>ORIGINAL ARTICLE</b>	135	Association of comorbidities with increasing severity of peripheral neuropathy in diabetes mellitus <i>Sachedina S, Toth C</i>
<b>BRIEF ARTICLE</b>	145	Diabetes-related impairment in bone strength is established early in the life course <i>Casazza K, Hanks LJ, Clines GA, Tse HM, Eberhardt AW</i>
	151	Vildagliptin-insulin combination improves glycemic control in Asians with type 2 diabetes <i>Kozlovski P, Foley J, Shao Q, Lukashevich V, Kothny W</i>
	157	Effect of treatment of overt hypothyroidism on insulin resistance <i>Nada AM</i>

**APPENDIX** I-V Instructions to authors

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## Status of autoimmune diabetes 20-years after generation of BDC2.5-TCR transgenic non-obese diabetic mouse

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### Abstract

Type 1 diabetes (T1D) is an autoimmune disease that results from the destruction of insulin-producing  $\beta$  cells by autoreactive T cells, leading to lifelong dependency on insulin therapy and increased risk of long-term cardiovascular complications. Here we take the opportunity of the 20<sup>th</sup> anniversary of the generation of the BDC2.5 TCR transgenic non-obese diabetic (NOD) mouse model, to provide a brief overview of the significant progress that has been made in understanding the role of T cells in the disease pathogenesis period. This included development of hundreds of reagents that block or even reverse new-onset disease by directly or indirectly controlling T cells. We also reflect on the sobering fact that none of these strategies has shown significant efficacy in clinical trials and discuss potential reasons hindering translation of the preclinical findings into successful therapeutic strategies and potential ways forward.

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**Key words:** Autoimmune diabetes; Immunotherapy; T cells; BDC2.5 T cells; Anti-CD3; Immunosuppression

**Core tip:** Our understanding of type 1 diabetes pathogenesis has significantly improved over the last three decades. We went from not knowing very little to acquisition of significant details about the role of the immune system and different T cell subsets in the disease process. The non-obese diabetic mouse model contributed and continues to contribute to our understanding of the disease process. This article pays tributes to the major role T-cells bearing -cell - specific T-cell receptors transgenic mouse played in shaping of our understanding of the disease process. We also divulge to briefly discuss current challenges facing development of a safe immunotherapy for the disease.

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### COMMENTARY ON HOT TOPICS

Diabetes is a heterogeneous metabolic disease caused by glucose intolerance and manifested clinically as hyperglycemia. Based on the underlying cause of the hyperglycemia, diabetes is divided into type 1 (T1D) and 2 (T2D). T1D is autoimmune in nature and results from the destruction of insulin-producing  $\beta$  cells by autoreactive T cells, leading to insulin deficiency and dependency on exogenous insulin to maintain glucose homeostasis. In contrast, T2D is a complex metabolic disorder associated with insulin resistance in peripheral tissues. Currently, there is no cure for either type of diabetes. In the interim, T1D is managed by multiple daily injections of insulin, whereas T2D is controlled by medications that improve insulin sensitivity and/or reduce glucose production by

the liver. Maintenance of glucose homeostasis, however, is challenging and most patients eventually develop fatal cardiovascular complications. Intensive efforts are therefore being directed toward development of cure or prevention strategies. Small animal models play profoundly important roles in these efforts, particularly in T1D research.

Small animal research in T1D began in earnest with the development and use of spontaneous and induced disease models in 1970s and 1980s. Among several T1D models, the non-obese diabetic (NOD) mouse became the most commonly used and favorite model soon after its development about 33 years ago<sup>[1]</sup>. The value of the NOD mouse in understanding the disease mechanism increased exponentially in the late 80s and early 90s following development of technologies that allowed engineering of the genome to generate mice bearing particular transgenes or lacking specific molecules to interrogate their roles in the disease process<sup>[2]</sup>. Consequently, more than 250 different genetically modified NOD mice were produced and characterized (<http://jaxmice.jax.org/find-mice/index.html>). Results of these efforts uncovered a wealth of information about the roles of various cell types and molecules in modulating T cells and established key cellular and molecular events in the disease process.

Of interest is that uncovering the role of T cells in autoimmune diabetes traversed several key steps that culminated in the generation of the NOD mouse bearing TCR transgenic T cells [reviewed in detail in by Haskins<sup>[3]</sup>. Considerable evidence accumulated in the early 1990s indicating a central role for T cells in mediating T1D in mice. These included demonstration that the disease development can be prevented by immunosuppressive agents that target T cells<sup>[4]</sup>, and by anti-CD4 and anti-CD8 antibody treatments<sup>[5,6]</sup>. Furthermore, the disease was shown to be transferrable to neonatal NOD mice and immunodeficient NOD-severe combined immunodeficiency mice (NOD-SCID) by adoptive transfer of T cells from spontaneously diabetic NOD donors<sup>[7]</sup>. A clearer picture of the role of T cells began to emerge with the generation of islet antigen-specific T cell clones. Several groups independently generated islet antigen-specific T cell clones capable of transferring the disease to susceptible recipients<sup>[4]</sup>. It was found that different T cell clones expressed different T-cell receptors (TCRs), suggesting for the first time that islet-specific T cells recognize several different islet antigens and pointing to the complexity of the disease. Among the well-characterized clones is the BDC2.5 clone, the TCR that was later used to generate the T-cells bearing-cell-specific T-cell receptors (BDC 2.5 TCR) transgenic (tg) mouse in 1993<sup>[8]</sup>. Thus, generation of T cell clones was crucial in cementing the role of T cell in the disease pathogenesis and the existence of diabetogenic T cells in autoimmune-prone hosts. Yet clones have limited value in providing details regarding the nature and *in vivo* action mechanisms of diabetogenic T cells. Among the pressing questions (some of which are still incompletely understood) are how autoreactive T cells escape negative selection, where they

reside in the periphery, what triggers them to become diabetogenic, and how they cause the disease. Diabetogenic T cells among the peripheral T cell repertoire are rare and the lack of appropriate reagents that permit their identification *in vivo* precluded addressing these questions directly *in vivo* in unmanipulated NOD mice. To overcome this problem, researchers generated TCR tg mice by using TCRs derived from generated clones. Among the widely used TCR transgenic mice in autoimmune diabetes is the BDC2.5 TCR tg mouse generated in 1993 by Katz *et al.*<sup>[8]</sup>, in which all T cells express the TCR $\alpha$  (V $\alpha$ 1) and  $\beta$  (V $\beta$ 4) chain genes from the BDC2.5 TCR CD4 T cell clone<sup>[9]</sup>. Unlike in wild type NOD mice, which harbor a diverse repertoire where autoreactive T cells are very rare and are difficult to track *in vivo*, all T cells in BDC2.5 tg mice recognize and respond uniformly to an elusive islet autoantigen [It was recently reported by two groups<sup>[10,11]</sup> that BDC2.5 T cells recognize peptides from chromogranin A (ChgA)]. Therefore, by studying T cells in BDC2.5 tg mice, the authors were able to track the behavior and fate of diabetogenic T cells *in vivo* and test hypotheses pertaining to roles of thymic selection, site of priming and peripheral activation of diabetogenic T cells, trafficking, and timing of response to islet autoantigens. Results showed that diabetogenic TCR can be produced in a large proportion of thymocytes in the TCR  $\alpha\beta$  tg mice, are positively selected without undergoing massive clonal deletion, and migrate to the periphery where they constitute the majority of the T cell repertoire. The model is still providing an important platform for *in vivo* dissecting of diabetogenic T cells, including roles of various molecules and cell types in modulating their pathogenicity. It has not only resulted in a wealth of information regarding pathogenesis of autoimmune diabetes, but also shed light on the immune system and autoimmunity.

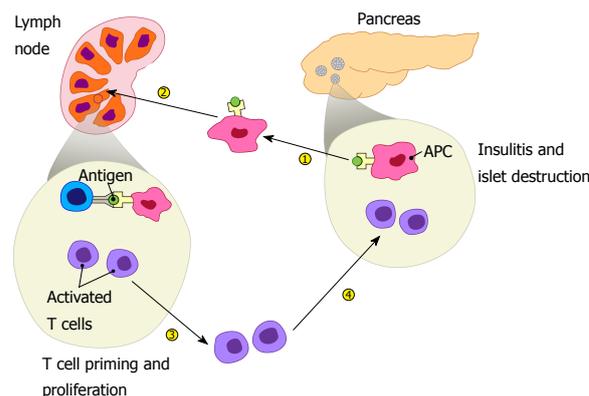
Tracking disease development in BDC2.5 TCR tg mice showed that initiation of the disease is highly regulated with two important checkpoints controlling the diabetogenic process. These two checkpoints are especially evident and synchronous in BDC2.5 tg mice. The autoreactive T cells appear to ignore the  $\beta$  cells for the first 2 wk of life. Soon after, BDC2.5 T cells abruptly invade the pancreatic islets resulting in insulinitis that progresses rapidly, with almost all islets heavily infiltrated by the age of 3 to 4 wk. Surprising at the time, however, was the observation that insulinitis in most BDC2.5 tg mice never progresses to full-blown diabetes. But when the BDC2.5 transgene is introduced into NOD-Rag-1 knockout mice, they do develop aggressive disease at a very early age. Failure of BDC2.5 TCR tg mice to develop full-blown disease in Rag-1-sufficient background was due to incomplete allelic exclusion of endogenous TCR $\beta$  chains, resulting in developing thymocytes that differentiate into regulatory T cells that oppose the pathogenic effect of diabetogenic T cells leading to standstill insulinitis. On the other hand, in the absence of the *Rag-1* gene all developing T cells bear the BDC2.5 TCR transgene, resulting in a pathogenic repertoire devoid of regulatory cells, inducing

a rapid onset of aggressive disease. The results provide critical hints of a major role for regulatory T cells in opposing the disease development. The synchronous development of the disease in BDC2.5 mice combined with other studies, including adoptive transfer of BDC2.5 T cells, led to the concept that immunoregulatory mechanisms exist at two check points, at the pancreatic draining lymph nodes and the islet itself, respectively. Breach of these checkpoints by diabetogenic T cells is clearly visualized in NOD mice by using adoptive transfer of BDC2.5 in appropriate hosts<sup>[12,13]</sup>. This paradigm is depicted in Figure 1. Subsequent studies revealed critical roles for regulatory T and B cells and various molecules involved in controlling the major checkpoints, and prevention and cure of the disease in the NOD mouse. Over the last two decades, vast numbers of molecules necessary for maintaining immunoregulatory mechanisms and others that facilitate their subversion have been identified. Targeting these molecules identified more than 250 interventions capable of preventing the disease in the NOD mouse. Some, like treatment with anti-CD3<sup>[14]</sup> and anti-CD20<sup>[15]</sup> reversed the disease in as many as 30%-50% of new-onset cases, raising hope of developing strategies to reverse disease in newly diabetic patients. Consequently, in the last few years, clinical trials have been conducted to test efficacy of several molecules including anti-CD3 and anti-CD20.

### Sobering reality facing translation of preclinical data into effective immunotherapeutics and ways forward

Translating immunotherapies found effective in preclinical studies into human therapies is proving challenging<sup>[16]</sup>, at least for now. Several high profile clinical trials including phase III have failed to demonstrate significant efficacy for all those tested<sup>[17,18]</sup>. The disappointing results in the clinic are forcing a retreat to drawing boards and generating second thoughts about whether the NOD mouse has surpassed its life expectancy as a research model and even the value of NOD mice in predicting and evaluating immunotherapy for T1D. It is easy to lay the blame on biologic differences between humans and mice, accentuated by more than 60 million years since their divergence into two species that differ in size, lifespan, and lifestyle (habitat/environment). The immune system in humans and mice, however, are generally quite similar, and with few notable exceptions, most paradigms translate well between them. Thus, the intangible efficacy of modalities such as anti-CD3 in humans is not entirely justified by biologic differences between the two species.

We argue that environmental factors play a dominant, if not the dominant role, in subverting therapeutic efficacy of modulators acting alone or in synergy with genetic factors<sup>[19,20]</sup>. This is acutely evident in the NOD mouse itself. For instance, the variability of anti-CD3 efficacy in reversing new-onset hyperglycemia ranges from about 30%-80% in newly diabetic NOD mice housed in the same facility<sup>[14,21]</sup> and mostly likely mice in the same cage responded differently. The low efficacy in NOD mice



**Figure 1** Pathogenesis of type 1 diabetes includes four major steps: islet autoantigens are picked up by antigen presenting cells from the pancreas, which then migrate to draining lymph nodes and present the autoantigens to autoreactive T cells, leading to their priming. Activated autoreactive T cells undergo proliferation, differentiation, and acquire homing molecules that direct them to the pancreas where they infiltrate the islets resulting in insulinitis and  $\beta$  cell destruction.

given the extremely small variations in their genetic makeup and exogenous influence of the environment suggests that treating the same mice under virtually identical conditions, the treatment would be successful only once out of at least two attempts. Applying the comparison to patients with markedly different genetic backgrounds, types of food, environment, and microbiota, the odds of success would be extremely low. Therefore, there is still much to be learned in the NOD mouse to uncover causes of variability on rate of disease onset, timing and response to treatment. In addition, understanding why females are more susceptible to disease than males<sup>[22-24]</sup> and why NOD mice housed in conventional facilities do not develop disease remains unclear<sup>[25]</sup>. It will also be important to understand why inactivation of molecules such as Fas death receptor or its ligand prevents disease in NOD mice<sup>[13,16,26-29]</sup>. Understanding mechanisms underlying these observations would provide important clues that could potentially facilitate the development of therapeutic strategies with high efficacy rates that are effective in both mice and men.

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## Trace elements in diabetic cardiomyopathy: An electrophysiological overview

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### Abstract

There is a growing body of evidence that Diabetes Mellitus leads to a specific cardiomyopathy apart from vascular disease and bring about high morbidity and mortality throughout the world. Recent clinical and experimental studies have extensively demonstrated that this cardiomyopathy causes impaired cardiac performance manifested by early diastolic and late systolic dysfunction. This impaired cardiac performance most probably have emerged upon the expression and activity of regulatory proteins such as  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase, ryanodine receptor and phospholamban. Over years many therapeutic strategies have been recommended for treatment of diabetic cardiomyopathy. Lately, inorganic elements have been suggested to have anti-diabetic effects due to their suggested ability to regulate glucose homeostasis, reduce oxidative stress or suppress phosphatases. Recent findings have shown that trace elements exert many biological effects including insulin-mimetic or antioxidant activity and in this manner they have been recommended as potential candidates for treatment of diabetes-induced cardiac complications, an effect based on their modes of action. Some of these trace elements are known to play an essential role as component of

enzymes and thus modulate the organ function in physiological and pathological conditions. Besides, they can also manipulate redox state of the channels *via* antioxidant properties and thus contribute to the regulation of  $[\text{Ca}^{2+}]_i$  homeostasis and cardiac ion channels. On account of little information about some trace elements, we discussed the effect of vanadium, selenium, zinc and tungstate on diabetic heart complications.

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**Key words:** Diabetic cardiomyopathy; Electrophysiology; Trace elements; Insulin-mimetic; Antioxidant

**Core tip:** Diabetic cardiomyopathy is one of the major causes of mortality in diabetic patients. Common cellular defects underlying the progressive cardiac complications of diabetes are reduction in the rate of contraction, low myosin ATPase activity, dysregulation of  $[\text{Ca}^{2+}]_i$  homeostasis and altered ionic currents. Accordingly, it is of critical importance to develop therapeutic strategies that will effectively inhibit diabetes induced fatal complications. In last decade, several trace elements have been suggested to improve performance of diabetic heart based due to their potential anti-diabetic and/or antioxidant activity. In this article the effects of trace elements on electrophysiological alterations of diabetic heart were discussed in detail.

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### INTRODUCTION

Cardiomyopathy, which develops independent of any major vascular disease, is one of the main complications

of diabetes resulting in a high percentage of morbidity and mortality. Although atherosclerotic vascular diseases occur frequently in diabetic conditions, a specific type of cardiomyopathy that results in impaired cardiac performance has been widely described in clinical and experimental studies<sup>[1-7]</sup>. In clinical aspect, diabetic cardiomyopathy is a disease which manifests itself particularly by early diastolic and late systolic dysfunction. As a matter of fact, elevated end-diastolic left ventricular (LV) pressure, reduced end-diastolic LV volume, impaired LV function in response to physiological stress and reduced LV filling rates in diabetic humans and animals are well-characterized<sup>[7-9]</sup>. These functional abnormalities of diabetic heart are likely to stem from multiple cellular defects such as reduction in the rate of contraction and relaxation, low myosin ATPase activity, myosin isoforms' shift from V1 (fast) to V3 (slow), deterioration of sarcoplasmic reticulum (SR) calcium uptake and reduction in glucose carrier (GLUT-4)<sup>[10,11]</sup>. Consistent with this, our reports and other studies have demonstrated prolonged periods of contraction and relaxation and in turn reduced tensile strength of rat papillary muscle in type 1 diabetes<sup>[12,13]</sup>. However, unchanged tensile strength despite slow left ventricular papillary muscle contraction and relaxation has been also suggested in experimental diabetes model in rats<sup>[3,4,14]</sup>. At cellular level prolongation of action potential (AP) duration has been consistently shown in diabetic hearts<sup>[3,4,15,16]</sup>. Significant alterations in the ionic currents that constitute AP configuration have been proposed as the main culprit of this prolongation, and indeed reduced transient outward ( $I_{to}$ ) along with smaller steady-state  $K^+$  currents (or  $I_{ss}$ ) have been reported<sup>[3-6,15,16]</sup>, despite unchanged  $Ca^{2+}$  currents<sup>[5,6]</sup>. Additionally, inward rectifier  $K^+$  current ( $I_{K1}$ ) has not been stated to have changed, but the delayed rectifier current ( $I_K$ ), thought to modulate late repolarization of AP, has decreased in diabetic ventricular cells<sup>[6,16]</sup>.

On the other hand, regulation of intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) is very critical for myocardium and has overriding impact on the contraction of heart. Therefore, diabetes induced abnormalities in cardiac contractility have been correlated with the intracellular  $[Ca^{2+}]_i$  changes<sup>[5, 6,12,14,17]</sup>. However, despite the plenty of data about dysregulated  $[Ca^{2+}]_i$  in diabetic myocardium, current findings are somehow controversial particularly in terms of the direction of change<sup>[14,18,19]</sup>. Nevertheless, amplitudes of  $Ca^{2+}$  transients recorded under electrical stimuli have been reported to be smaller, while their time to peak and decay were mostly longer<sup>[5,6,19-22]</sup>. Therefore, it is most likely that diabetic cardiac dysfunction arises due to changes in expression and/or activity of cellular mechanisms that regulate  $[Ca^{2+}]_i$  during cardiac cycle. This possibility has been widely studied over years and indeed a significant decrease has been found in expression of regulatory proteins such as  $Na^+/Ca^{2+}$  exchanger NCX (NCX), sarcoplasmic reticulum  $Ca^{2+}$  ATPase (SERCA), ryanodine receptor (RyR) and phospholamban (PLB), along with reduced activity of NCX and SERCA<sup>[11,18,20,22-25]</sup>.

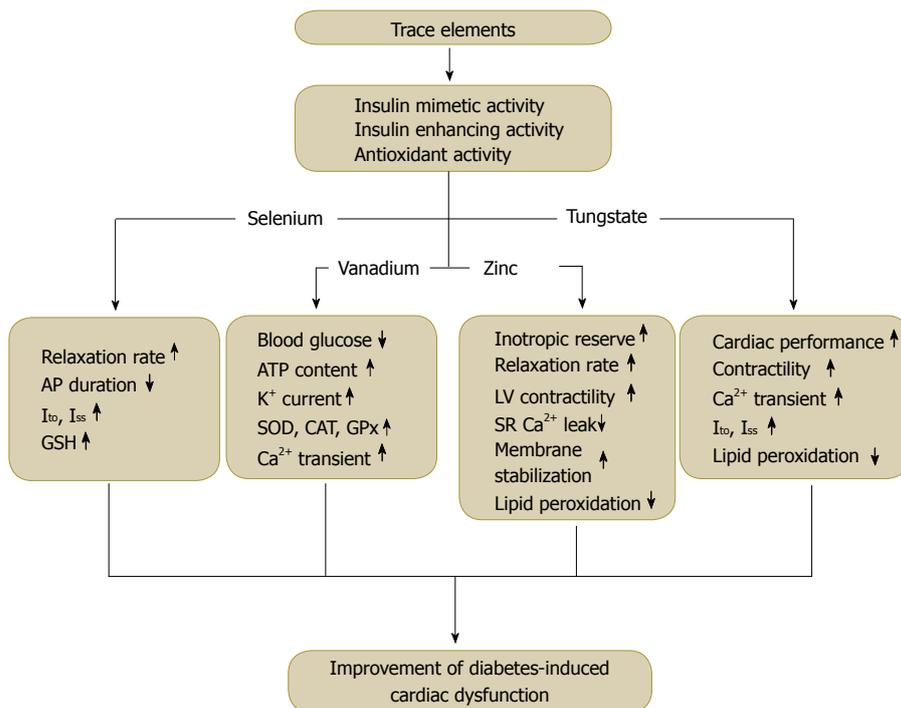
However diabetes is characterized by complexity; it likely involves activation of different pathways leading to abnormal  $[Ca^{2+}]_i$  homeostasis and thus contractile dysfunction. For example, currently it is clearly evident that reactive oxygen species (ROS) and resultant oxidative stress is involved in the pathogenesis of diabetic cardiomyopathy. Hyperglycemia leads to generation of superoxide radicals from both mitochondrial (*via* oxidation of glucose) and non-mitochondrial sources (xanthine oxidase, nitric oxide synthase and NADPH-oxidase)<sup>[26]</sup>.

## DIABETES AND TRACE ELEMENTS

In recent years many inorganic elements have been recommended as dietary supplement to alleviate the impaired insulin metabolism in diabetic patients<sup>[5,26-30]</sup>. Being essential or not, trace elements have been identified for long time as potential candidates for treatment or to mitigate severity of complications of some metabolic disorders including diabetes (Figure 1). Activation of insulin receptor signaling, antioxidant properties or inhibition of phosphatases have been depicted as potential ways of action in modulating glucose homeostasis and preventing organ damage<sup>[26,29,31]</sup>. On the other hand, cardiac complications have been progressively becoming the main cause of death among diabetics due to the improvements in the treatment of diabetic complications with non-cardiac origin. Accordingly, it is of critical importance to develop therapeutic strategies that will effectively inhibit diabetes induced fatal cardiac disorders. Consistently, trace elements, some of which are involved in metabolism as essential components of enzymes, have also been suggested to improve the reduced cardiac performance in diabetic heart due to their presumed insulin-mimetic or antioxidant activity<sup>[3,5,28,32]</sup>. Furthermore, recent studies have demonstrated that the underlying mechanism of this improvement is due most probably to restoration of abnormal  $[Ca^{2+}]_i$  homeostasis and cardiac ion channels. Despite the limited number of studies, it is evident that either insulin-mimetic or antioxidant, trace elements are capable of modulating expression and/or redox status of ion channels and  $[Ca^{2+}]_i$  regulating proteins<sup>[33-36]</sup>. Of the inorganic or trace elements currently known; vanadium, selenium, zinc and tungstate were discussed in this review, since the effects of other inorganic elements on diabetic cardiac complications have not been well-documented yet.

### Selenium

Selenium was first discovered by Berzelius in 1818. This Swedish chemist named that new chemical element after Selene, the Greek goddess of the moon. Selenium is an essential trace element in man and animals, since it is an integral part of selenium dependent glutathione peroxidase<sup>[36]</sup>. In humans and experimental studies, selenium deficiency has been suggested to result in increased risk of various pathologies including cardiovascular diseases<sup>[37]</sup>. Particularly, selenium deficiency results in Keshan disease, which is a special type of cardiomyopathy caused by di-



**Figure 1** The summarized effects of trace elements on diabetes-induced cardiac complications. AP: Action potential; LV: Left ventricle; SR: Sarcoplasmic reticulum; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GSH: Glutathione.

etary inadequacy of selenium and responds to treatment with sodium selenite<sup>[38]</sup>. Furthermore, adequate selenium intake is required for optimal activity of some key antioxidant enzymes, including glutathione peroxidases and thioredoxin reductases, which act to prevent free radical damage to various cells<sup>[39,40]</sup>. As a result of its protective role against oxidative stress, selenium raised considerable expectations for the prevention of cardiovascular diseases including diabetic cardiomyopathy. It appears to have insulin-like effects when administered *in vivo*<sup>[41]</sup>. In fact, several reports have suggested that plasma glucose levels were significantly though not completely improved in the diabetic rats treated with selenite in different ways either orally or *via* injection<sup>[3,42-44]</sup>. Interestingly, similar to vanadium, selenite decreases plasma glucose levels in hypoinsulinemic rats without an accompanying correction of the insulin levels<sup>[3,42]</sup>. Nevertheless, the reduced cardiac performance characteristics of diabetic rats such as left ventricular developed pressure (LVDP), positive dP/dt (+dP/dt) and negative dP/dt (-dP/dt) have been found to be reversed with selenite treatment<sup>[42,45]</sup>.

The effects of sodium selenite treatment on mechanical and electrical properties of diabetic heart have been also studied in detail. Ayaz *et al.*<sup>[3]</sup> demonstrated that selenium supplementation for 5 wk was capable of reducing the prolonged peak time and relaxation of electrically stimulated papillary muscle twitch in diabetic rats, although no change was reported between peak tension of the experimental groups. Additionally, the prolonged AP duration, which is a typical characteristic of diabetic heart, was shown to be restored after the treatment. In the same study, the major repolarizing currents of AP,  $I_{to}$

and  $I_{ss}$ , were lower in untreated diabetic cardiomyocytes while selenium achieved an apparent increase in treated-diabetics. However, plasma insulin levels didn't increase significantly despite the long-term administration of selenium<sup>[3]</sup>. Although the precise mechanism of this beneficial effect is not known currently, it is likely that oxidative stress, which has been suggested to involve in the etiology of diabetes-induced downregulation of ion channels, is balanced through selenite-mediated augmentation of glutathione levels, and resultant enhancement of endogenous antioxidant defense mechanisms<sup>[3,5,28,46]</sup>. Additionally, oxidative species have been recognized to modulate  $K^+$  channels and cellular  $Ca^{2+}$  regulation, notably *via* redox modifications of key amino acid residues involved in the function of ion channels and transporters<sup>[33-35,47]</sup>. Therefore, it is most likely that selenium may achieve recovery of impaired cardiac performance and altered  $K^+$  currents of diabetic cardiomyocytes *via* restoration of the oxidized groups of ion channel proteins.

### Vanadium

Vanadium is a trace element that exists naturally in water and soil and found in different physiologically active oxidation states<sup>[29]</sup>. Although the exact physiological actions of vanadium are not known yet, it is supposed to be necessary for the body as a trace element since its deficiency has been suggested to result in a variety of side-effects<sup>[29,48]</sup>. In addition to reproductive problems and skeletal abnormalities observed in case of deficiency, vanadium is likely to have a significant role in thyroid, iron, glucose and lipid metabolism<sup>[29,49]</sup>. The total vanadium content of the body has been estimated to be approximately 200  $\mu\text{g}$ <sup>[29,50]</sup>. The

beneficial effects of vanadium have been widely studied in diabetic conditions and speculated to exert insulin-mimetic activity through a specific tyrosine kinase receptor or to annihilate free radicals due to its antioxidant activity<sup>[51-55]</sup>. Therefore, the potential use of vanadium in the treatment of diabetic complications including cardiomyopathy has been assessed and indeed its hypoglycemic effect along with reversal of functional abnormalities has been clearly demonstrated by several studies<sup>[28,56-59]</sup>.

In the last decade, the effect of vanadate compounds on impaired performance of diabetic heart has been investigated in a large number of studies that have shown significant improvement in diabetes with vanadate treatment<sup>[28,56]</sup>. Ozcelikay *et al.*<sup>[60]</sup> reported that vanadate treatment was capable of normalizing blood glucose and serum thyroid hormone levels, despite the fact that serum insulin level of diabetic animals was not corrected significantly. Moreover, vanadate treatment resulted in normalization of mechanical alterations and reversed the decreased responsiveness of diabetic atria to isoprenaline in spontaneously-beating preparations from diabetic rats. Similarly Heyliger *et al.*<sup>[56]</sup> assessed the impact of vanadate on cardiac performance in diabetic female rats and found that vanadate was capable of restoring blood glucose but not insulin levels when administered for a 4-wk period to the diabetic rats. In the same study, vanadate treatment prevented the decline in cardiac performance due to diabetes. Organic vanadium complex, bis (maltolato) oxovanadium (IV) was also reported to correct working heart parameters such as LVDP and  $\pm$  dP/dT values in streptozotocin-induced diabetic rats, which indicated the protective effect of vanadium derivatives against heart dysfunction associated with type 1 diabetes in rats<sup>[61]</sup>. Consistently, decreased peak  $\pm$  dP/dt and reduced cardiac efficiency of diabetic hearts were fully restored while myocardial ATP content significantly increased by vanadate administration<sup>[62]</sup>. These results, thus, indicate that the normalizing effect of vanadate on diabetes can contribute to the prevention of cardiac changes observed at the early and late stages of diabetes.

Taking the central role that  $\text{Ca}^{2+}$  plays in cardiac electrical and mechanical activity, it is likely to suggest that the beneficial effects of vanadate entail modulation of  $\text{Ca}^{2+}$  regulation in diabetic cardiomyocyte. In fact Clark *et al.*<sup>[28,29]</sup> demonstrated that tea-vanadate treatment had normalized the contractile response of diabetic cardiomyocytes and ameliorated the  $\text{Ca}^{2+}$  transients to an extent equal to or better than that of insulin treated diabetic animals. It is an effect that were attributed to the alleviated glycemic status because tea/vanadate decoction has been shown to restore glycemic status effectively in rodent models of both Type I and Type II diabetes mellitus<sup>[63,64]</sup>. Interestingly, tea/vanadate decoction exhibited vastly improved glycemic status that could persist beyond treatment period<sup>[63]</sup> and relieved diabetic animals from non-specific side-effects of vanadate or its analogues to other organs in the body<sup>[65,66]</sup>. Vanadate also mimics the enhancing effect of insulin on cardiac  $\text{K}^{+}$  currents

(particularly  $\text{I}_{\text{to}}$ ) in sucrose-fed rats with 3-4 wk treatment or 5-6 h incubation of myocytes, an effect suggested to arise due probably to synthesis of new channels<sup>[67]</sup>. Hence, although we don't have such data, it is tempting to speculate that vanadate is likely to shorten AP duration in diabetic myocardium and thereby modulate ventricular repolarization and dispersion of repolarization that have been shown to be a major cause of cardiac arrhythmias in diabetes mellitus<sup>[68]</sup>.

Vanadate is thought to act *via* insulin-mimetic and/or insulin-enhancing action<sup>[69]</sup> or through activation of lipid signaling mechanisms like the phosphatidylinositol pathway<sup>[54]</sup>. It can also scavenge free radicals<sup>[55]</sup>, and accordingly, vanadate administration has been reported to decrease oxidative damage remarkably in the diabetic heart<sup>[70]</sup>. Therefore, the beneficial effect of vanadate on diabetes-induced cardiac dysfunction may stem from its ability to serve as a scavenger of free radicals<sup>[27,54,71]</sup>. With vanadium treatment, glutathione peroxidase, catalase and superoxide dismutase levels have been corrected to near normal values in diabetic rats<sup>[54,55]</sup>. However, one another study attributed some of these effects to vanadate's ability to prevent diabetic hypothyroidism<sup>[60]</sup>. In conclusion, despite the plenty of findings that provide evidences for improving effect of vanadium on diabetic heart dysfunction due most probably to its insulin-mimetic and/or antioxidant action, further studies are needed to fully elucidate the molecular mechanism of these beneficial effects.

## Zinc

Zinc is an essential trace element that is critical in maintaining cellular functions since it is the cofactor of numerous enzymes and transcription factors<sup>[26,72]</sup>. In normal cellular physiology, much of the intracellular zinc is found in protein bound form and participates in phosphorylation/dephosphorylation cascades. Besides, it acts as a second messenger in the signaling system<sup>[73]</sup> and affects the redox status of the cell. Thus, in particular conditions zinc can either enhance the cell's antioxidant capacity or trigger the production of reactive oxygen species<sup>[26,74]</sup>. Consistent with this, Zn deficiency has been suggested to result in increased oxidative damage in multiple organs including the heart<sup>[75-77]</sup> due to the decreased cardiac antioxidant capacity<sup>[76,78]</sup>.

It has been demonstrated that Zn deficiency induced by low concentrations of Zn in drinking water<sup>[79]</sup> and by Zn chelators increases the likelihood of diabetes in humans and animals<sup>[80]</sup>. Therefore, it is likely that Zn deficiency can be a risk factor for the development of diabetes, and in reciprocal manner, diabetes itself can dysregulate Zn homeostasis. Indeed, systemic Zn deficiency has been associated with the high incidence of diabetic cardiovascular complications<sup>[72,79,81,82]</sup>. The potential role of zinc in the protection of diabetic patients from coronary heart disease has been investigated in a recent clinical trial in which serum zinc level was inversely proportional to cardiovascular complications<sup>[83]</sup>. Measurements of cardiac function have demonstrated that Zn is

capable of improving left ventricular systolic and diastolic function. Moreover, inotropic reserve of left ventricle was enhanced in the heart of the diabetic mice treated with Zn compared to that without Zn, which implicates alleviated cardiac function with Zn supplementation<sup>[30]</sup>. Wang *et al.*<sup>[84]</sup> observed lower  $\pm$  dP/dtmax, suggesting reduced LV contractility along with slowing of relaxation in the diabetic mice, which both improved following Zn supplementation to near control levels. Furthermore, Zn ameliorated the diabetes-induced catecholamine desensitization markedly, which was quantified by measure of augmentation of dP/dtmax after  $\beta$ -adrenergic stimulation. Thus, they concluded that zinc is capable of improving both basal and stimulated LV function as well as inotropic reserve in diabetic hearts.

On the other hand, incomplete relaxation and reduced contractile function which were more prominent as pacing frequency increased has been reported in diabetic cardiomyocytes, but these changes were significantly restored by extracellular Zn exposure<sup>[8]</sup>. These findings provide evidences that suggest zinc administration could be a possible long term management regimen for incomplete relaxation and diastolic dysfunction associated with diabetic cardiomyopathy. In addition, extracellular zinc ion has been proposed to compete with  $\text{Ca}^{2+}$  for the cardiomyocyte L-type  $\text{Ca}^{2+}$  channel and, the release of SR Zn through the RyR also appears to be regulated similarly to that of SR  $\text{Ca}^{2+}$ <sup>[85-87]</sup>. Moreover, extracellular Zn exposure could lower the open probability of RyR and presumably reduces SR  $\text{Ca}^{2+}$  leak through the RyR, which has been shown to be elevated in hyperglycemic conditions<sup>[88,89]</sup>. Given these results, it is likely that Zn exerts a competitive effect on  $\text{Ca}^{2+}$  regulatory mechanisms and modulates cardiomyocyte function.

Although the cellular and molecular mechanisms responsible for zinc-induced protection against diabetic cardiomyopathy has not been fully understood yet, zinc-binding protein metallothionein (MT) has been proposed to play a role in cellular defence against oxidative stress associated with diabetic cardiomyopathy<sup>[72,84,90]</sup>. Indeed, Zn supplementation provides significant protection of the heart from oxidative stress. Zn has been demonstrated to act as an antioxidant through participation in SOD and thioredoxin enzymatic and chelator activities, stabilizing cell membranes, and inhibiting lipid peroxidation<sup>[26,74,91]</sup>. Additionally, the relationship between Zn and diabetes appears to be complex. Several complications of diabetes have been supposed to be related to increased intracellular oxidants and free radicals associated with decreases in intracellular Zn and in Zn-dependent antioxidant enzymes<sup>[92]</sup>. Moreover, Zn is suggested to be important for the normal conformation, secretion and function of insulin<sup>[26,30]</sup>.

All these observations strongly support the notion that Zn deficiency occurs in diabetic subjects<sup>[82]</sup> and Zn supplementation may improve cardiac dysfunction or damage in these patients due to its systemic antioxidant capacity or modulation of the cellular ionic mechanisms.

However, the understanding of molecular mechanisms that involve in Zn related changes in diabetic heart deserves further investigation.

### Tungstate

Over the past decade, sodium tungstate ( $\text{Na}_2\text{WO}_4$ ), which chemically resembles vanadium has become a molecule of interest, since it has a relatively low toxicity and it has been suggested to have antidiabetic activity in experimental studies<sup>[93-96]</sup>. Although numerous studies have demonstrated the efficacy of tungstate as an antidiabetic agent in various models of experimental diabetes, only few of them have investigated whether it can improve cardiac performance of diabetic heart as well. One of these studies performed by Nagareddy *et al.*<sup>[31]</sup> has assessed cardiac function by measuring left ventricular pressure, the rate of contraction and the rate of relaxation. An apparent cardiac dysfunction has been shown in untreated diabetic rat hearts, which exhibited an inability to respond to the increase in left atrial filling pressure. However, the treatment of diabetic rats with tungstate has improved LVP, + dP/dt, and - dP/dt, particularly at higher filling pressures.

On the other hand, recently we have studied the cellular mechanism of that beneficial effect of sodium tungstate on diabetic myocardium at cellular level. We demonstrated that long-term sodium tungstate treatment was capable of ameliorating the amplitude of shortening and associated  $\text{Ca}^{2+}$  transients of diabetic cardiomyocytes, although it didn't improve the rate of relaxation in either traces. Moreover, we showed depressed  $I_{\infty}$  and  $I_{ss}$  in diabetic cardiomyocytes which were recovered significantly by tungstate administration that might be accomplished due to its antioxidant property<sup>[5]</sup>. This finding is important because diminished potassium currents and thus prolonged action potential in ventricular cells have been suggested to increase the likelihood of arrhythmia in diabetic patients<sup>[4,33,67,97]</sup>. Hence, tungstate administration is likely to reduce this propensity in diabetic patients.

The underlying mechanism of these beneficial effects has been mostly attributed to antioxidant or insulin-like activity of tungstate. Because hyperglycemia leads to abnormal increase of ROS production<sup>[5,11,35]</sup> that have been recognized to be capable of modulating  $\text{K}^+$  channels and  $[\text{Ca}^{2+}]_i$  regulation due to redox modifications of key amino acid residues involved in the function of intracellular and plasma membrane ion channels and transporters<sup>[33,35]</sup>. In fact, tungstate treatment was associated with significant reduction of lipid and protein oxidation levels in treated-diabetic rats, a finding that further supports this hypothesis. Insulin-mimetic or insulin-enhancing activity of tungstate is less likely since we didn't observe a remarkable change either in insulin or blood glucose levels after supplementation<sup>[5]</sup>. Contrary to this, some investigators have reported increased insulin and/or decreased glucose levels that might arise from very high level of tungstate they administered, which may cause side effects<sup>[98]</sup>.

## CONCLUSION

Diabetic cardiomyopathy, one of the major causes of mortality in diabetic patients, is associated with progressive contractile dysfunction. Therefore, it is crucial to develop therapeutic strategies that will effectively inhibit diabetes-induced fatal complications of the heart. Among the various therapeutic strategies, the restoration of glycemic status by insulin-enhancing or insulin-mimetic agents can be useful in the prevention of cardiomyopathy in diabetic patients.

In the last decade, several inorganic compounds such as selenium, vanadium, zinc and tungstate have been suggested to improve cardiac performance in diabetic heart based on its potential anti-diabetic and/or antioxidant activity. Some of these trace elements are known to play an essential role as components of enzymes and thus modulate the organ function in physiological and pathological conditions. Current findings clearly demonstrate that diabetic cardiomyopathy leads to ventricular dysfunction due to altered ionic homeostasis in myocytes which results in defective excitation-contraction coupling of myocardium and, trace element supplementation can prevent these changes and thus ameliorate the diminished cardiac function. Therefore, they may have a potential therapeutic use in preventing diabetic cardiomyopathy, although further investigations and substantial efforts are needed to elucidate the underlying mechanism of their beneficial effect. Furthermore, prior to clinical trials, the question whether they have side effects or not should be addressed unequivocally.

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## Biomolecular basis of the role of diabetes mellitus in osteoporosis and bone fractures

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**Core tip:** The physical complications due to diabetes mellitus are not limited since there have been going research to elucidate the relation of other diseases with diabetes mellitus (DM). Osteoporosis is one of the complicated diseases of human that may be linked with DM through different networks in the body. In this review a precise relationship has been made between DM and osteoporosis through a broad range of biophysical pathways.

### Abstract

Osteoporosis has become a serious health problem throughout the world which is associated with an increased risk of bone fractures and mortality among the people of middle to old ages. Diabetes is also a major health problem among the people of all age ranges and the sufferers due to this abnormality increasing day by day. The aim of this review is to summarize the possible mechanisms through which diabetes may induce osteoporosis. Diabetes mellitus generally exerts its effect on different parts of the body including bone cells specially the osteoblast and osteoclast, muscles, retina of the eyes, adipose tissue, endocrine system specially parathyroid hormone (PTH) and estrogen, cytokines, nervous system and digestive system. Diabetes negatively regulates osteoblast differentiation and function while positively regulates osteoclast differentiation and function through the regulation of different intermediate factors and thereby decreases bone formation while increases bone resorption. Some factors such as diabetic neuropathy, reactive oxygen species, Vitamin D, PTH have their effects on muscle cells. Diabetes decreases the muscle strength through regulating these factors in various ways and ultimately increases the risk of fall that may cause bone fractures.

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### INTRODUCTION

Osteoporosis (OP) has become an alarming health problem through the entire world and about 200 million people in the world are under the threat of this deleterious health problem<sup>[1]</sup>. Although OP is often described as a silent disease because it is typically asymptomatic until a fracture occurs, the disease negatively and significantly impacts morbidity and mortality as it can lead to severe pain, deformity, disability, and death<sup>[2]</sup>. The signs of OP are deterioration of the microstructure of bone specifically at trabecular sites including vertebrae, ribs and hips, culmination in fragility fractures, pain and disability<sup>[2,3]</sup>. The occurrence of OP is prevalent among the aging women than the aging men although corticosteroid treatment, intake of excessive alcohol, cigarette smoking, low calcium intake and hypogonadism may be the secondary cause<sup>[1,2]</sup>.

Like osteoporosis, diabetes mellitus is a pandemic and a chronic metabolic disorder with substantial morbidity

ity and mortality, characterized by the presence of high blood glucose<sup>[2,4,5]</sup>. According to the report (September 2012) of the World Health Organization (WHO) about 374 million people in the world are under the threat of this deleterious health problem<sup>[6]</sup>. Under chronic condition DM adversely affects the different parts of the body including bone, nerve, muscles, retina of the eyes, cardiovascular system and nephron of kidney<sup>[4]</sup>. The effects of DM on bone cell are very complex and several investigations have been conducted to explore the exact mechanisms through which DM induces osteoporosis and bone fractures and all the investigations have come to the end with few findings<sup>[6]</sup>. The exact mechanism of diabetes mellitus (DM) induced osteoporosis is almost unknown but it is plausible that, patients with DM have increased rate of osteoporosis and bone fractures<sup>[3,7-10]</sup>. Hyperglycemia may induce osteoporosis and bone fractures through exerting its effects on bone cells and muscle cells through different possible pathways. This review has explained the possible molecular mechanisms through which DM may induce osteoporosis and bone fractures.

## EFFECT OF DIABETES MELLITUS ON BONE CELLS

The bone mainly comprise of three basic types of cells osteoblast, osteocyte and osteoclast<sup>[11]</sup>. Osteoblasts commonly called bone-forming cells which derived from the osteoblast progenitor cells, participate in mineralization and are unable to multiply<sup>[11]</sup>. Osteocytes are mature osteoblast which no longer secretes matrix, participates in nutrient/waste exchange *via* blood and unable to divide. Osteoclasts are cells that derive from the macrophage-monocyte cell lineage and participate in bone resorption<sup>[1,11]</sup>.

### Osteoblast

Osteoblast originates from the mesodermal progenitor cell and among the three basic types of bone cells it plays a crucial role in bone formation. Binding of different types of growth factors and hormones including bone morphogenetic protein (BMP), Wnt, transforming growth factor- $\beta$  (TGF- $\beta$ ), parathyroid hormone (PTH), platelet derived growth factors (PDGFs), fibroblast growth factors (FGF) with their receptors expressed on the cell surface of mesodermal progenitor cells (also known as mesenchymal stem cells) induce the activation of different types of transcription factors responsible for osteoblast differentiation, maturation and survival<sup>[1,12]</sup>.

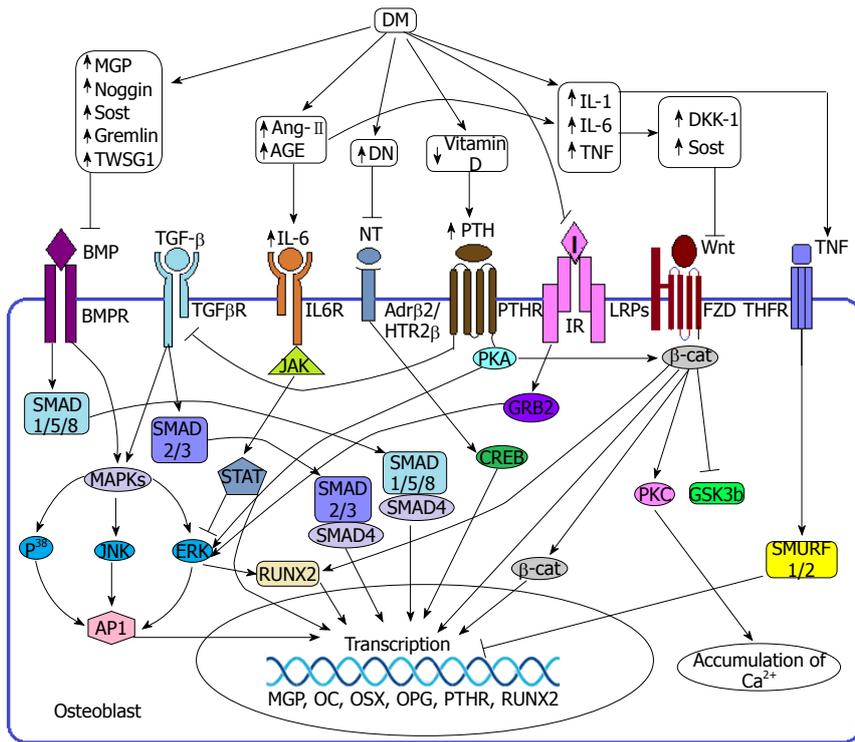
BMPs are the members of TGF- $\beta$  superfamily and known to be a potent inducer of osteoblast formation and thereby increase collagen synthesis and decrease collagenase-3 production<sup>[1,13]</sup>. There are several types of BMP proteins and among them BMP-2, BMP-4, BMP-5, BMP-6 and BMP-7 have strong capacity in osteogenesis<sup>[14]</sup>. BMP-2 and BMP-6 induce osteoblast formation and chondrocyte proliferation<sup>[14,15]</sup>. BMP-4 could participate in endochondral ossification<sup>[16,17]</sup> and BMP-7 induces the expression of markers including ALP activity and

accelerated calcium mineralization which are required for osteoblast differentiation<sup>[14]</sup>. But BMP-3 has adverse effect on osteoblastogenesis<sup>[14]</sup>. BMP signaling has been identified as the major signaling molecules in the pre osteoblast because the binding of BMPs to its receptors (BMPRs) induce phosphorylation of SMADs proteins specially SMAD-1, SMAD-5 and SMAD-8 (Figure 1). SMADs then in turn directly activate the SMAD binding element (SBE) through the SMAD depended pathway and thereby induces the transcription of corresponding genes. On the non SMAD depended pathway BMPRs directly activate MAPK and then in turn activate the particular genes through inducing runt related transcription factor 2 (RUNX2) or activator protein 1 (AP-1)<sup>[14,18,19]</sup>.

Wnt is the member of highly conserved secreted glycoprotein family, rich in cystein residue and are divided into two classes: canonical Wnts (wnt1, wnt3a) and non-canonical Wnts (wnt5a). Binding of canonical Wnts with frizzled (FZD) and LDL receptor related proteins (LRPs) promotes: the phosphorylation and inactivation of *glycogen synthase kinase 3 beta* (GSK3b), prevents the degradation of  $\beta$ -catenin ( $\beta$ -cat) as well as subsequent translocation of  $\beta$ -cat in the nucleus for binding with the target genes (Figure 1). Binding of non-canonical wnts with FZD receptor promote the activation of heterotrimeric G proteins in order to enhance the deposition of intracellular calcium ion ( $\text{Ca}^{2+}$ ) through protein kinase C (PKC) mediated pathway or induce the formation of the cytoskeleton *via* Rho/c-Jun N-terminal kinase dependent mechanism<sup>[18,19]</sup>.

TGF- $\beta$  signaling is important for the regulation, proliferation and commitment to the osteoblastic lineage of MSC. Binding of TGF- $\beta$  with its receptor TGF $\beta$ R regulates the expression of target genes through two possible pathways: canonical and non-canonical. In the canonical or smad dependent pathway activated TGF $\beta$ R promotes the phosphorylation of R-SMADs (SMAD-2, 3) and thereby activate the target genes through SMAD-4 mediated signal transduction (Figure 1). In the non-canonical or non smad dependent pathway activated TGF $\beta$ R promotes the expression of responsive genes through MAPK, P38, ERK mediated signal transduction pathway<sup>[12,14]</sup>.

Immunohistochemical analysis revealed that the periosteum and bone are linked with the sympathetic, sensory and the glutaminergic nervous system specifically the growth plate and the metaphysis of long bones are more exposed to the neural network. Close contact of the nervous system with the bone cells, strongly implying a physiological role of neural signal on bone health<sup>[20,21]</sup>. In addition, osteoblast has been reported to express  $\beta$ -2 adrenergic receptors ( $\beta$ 2AR) and 5-hydroxytryptamine receptor (5HTR) for several neurotransmitters including serotonin and norepinephrine<sup>[21,22]</sup>. An *in vivo* experiment showed that 5HTR 2 $\beta$  facilitate osteoblast recruitment and proliferation and the absence of this receptor leads to osteopenia<sup>[22]</sup>. Binding of neurotransmitter with particular receptors activates the transcription factor CREB and ultimately induces the gene for osteoblast prolifera-



**Figure 1 Diabetes mellitus induced regulation of osteoblast.** During healthy condition bone morphogenetic protein (BMP), transforming growth factor  $\beta$  (TGF- $\beta$ ), Wnt, insulin and neurotransmitter signaling are mandatory to the osteoblast for its normal functioning and survival. Binding of BMP with its receptor (BMPR) activates the corresponding gene through (a) Smad depended pathway: which requires the SMADs protein (SMAD 1/5/8) or (b) non smad depended pathway: in which activates RUNX2 or AP-1 through MAPK-ERK mediated pathway. Wnt-Frizzled pathway positively regulates gene expression through  $\beta$ -catenin or RUNX2 mediated pathway and Calcium accumulation through PKC mediated pathway. TGF- $\beta$  is also a positive regulator of osteoblast function and exerts its effect on the respective gene through SMAD 2/3 depended pathway or MAPK-ERK mediated pathway. Peripheral nerve exposure to the osteoblast signals through the adrenergic receptor 2  $\beta$  (Adr $\beta$ 2) or 5HTR induced pathway. Binding of neurotransmitters on the Adr $\beta$ 2 or 5HTR receptor activates ERK or CREB to induce the expression of osteoblastic gene. Insulin is a beneficial factor of bone formation and it exerts its effect through GRB2-ERK mediated pathway. During diabetes mellitus (DM), hyperglycemia may induce the expression of several BMP inhibitors including MGP, Noggin, Sost, Gremlin, TWSG1 as well as several Wnt inhibitors including DKK-1, Sost. DM also induces the production of different proinflammatory cytokines including interleukin 6 (IL-6), IL-1, AT-2 and TNF which negatively regulates osteoblast functioning. Binding of IL-6 with its receptor IL-6 receptor (IL6R) sequesters ERK pathway as well as induce the gene to transcribe several inhibitors including MGP, OPG, OSX. DM induced DN limits the nerve signaling through damaging the peripheral nerves. TNF binding with TNFR induce SMURF1/2 and thereby inhibit the transcription process. DM also reduces the production of vitamin D which in turn induces the secretion of parathyroid hormone (PTH). PTH binding with PTH receptor (PTHR) inhibits TGF- $\beta$  signaling through inhibiting TGF $\beta$  receptor (TGF $\beta$ R) although PTHR activates  $\beta$ -Cat and ERK pathways. DM induced IR (type-2DM) or insulin deficiency (Type-1DM) also limits insulin mediated bone formation. TNF: Neurotransmitter; HTR2 $\beta$ : 5-hydroxytryptamine receptor 2  $\beta$ ; I: Insulin; IR: Insulin receptor; LRP: Low density lipoprotein receptor related protein; FZD: Frizzled; TNF: Tumor necrosis factor; TNFR: TNF receptor; JAK: Janus kinase; STAT: Signal transducers and activators of transcription; AP-1: Activator protein 1; ERK: Extracellular signal regulated kinase; MAPK: Mitogen activated protein kinase; RUNX2: Runt related transcription factor 2; PKA: Protein kinase A; PKC: Protein kinase C;  $\beta$ -cat:  $\beta$  catenin; GSK3b: Glycogen synthase kinase 3b; SMURF: SMAD ubiquitylation regulatory factor; MGP: Matrix gla protein; OC: Osteocalcin; OSX: Osterix; OPG: Osteoprotegerin; DKK-1: Dickkopf related protein 1; Sost: Sclerostin; TWSG1: Twisted gremlin; Ang-II: Angiotensin-II; AGE: Advance glycation end product; GRB2: Growth factor receptor bound protein.

tion through AP1 activation<sup>[18]</sup>(Figure 1).

Elevated secretion of PTH has been reported to sequester osteoblast differentiation and activation. Attachment of PTH with its receptor PTHR activates protein kinase A (PKA) and extracellular signal regulated kinase (ERK) and ultimately induces the expression of matrix gla protein (MGP) on osteoblast which is a potent inhibitor of BMP signaling<sup>[1,14]</sup>. PTH binding also drives internalization of PTHR-TGF $\beta$ R complex, which attenuates TGF- $\beta$  signaling in bone development<sup>[14]</sup>.

Several extracellular, intracellular and transcriptional BMP inhibitors such as matrix gla protein (MGP), Noggin, dickkopf-related protein 1 (DKK-1), Sclerostin, Gremlin, Ski, Smurf-1, Smurf-2, twisted gastrulation (Twsg1), Interleukin 6 (IL-6) and TNFs have been

identified in the down regulation of BMP and TGF- $\beta$  signaling pathways and ultimately suppress osteoblast function<sup>[1,13,14,23,24]</sup>. MGP is the member of mineral binding  $\gamma$ -carboxyglutamic acid containing protein family that directly and indirectly sequesters mineralization of bone cells. In the direct effect it acts as a part of a complex with  $\alpha$ -2-HS glycoprotein and in the indirect effect it inhibits the binding of BMP-2 with its receptor expressed on the osteoblast precursors<sup>[1]</sup>.

Diabetes mellitus (DM) not only induces the over-expression of DKK-1<sup>[25,26]</sup> Sclerostin<sup>[27,28]</sup> Gremlin<sup>[29,30]</sup> PTH<sup>[31]</sup> angiotensin II (Ang-II)<sup>[32]</sup> IL-6<sup>[33]</sup> and TNFs<sup>[33-35]</sup> but also sequesters the over expression of Vitamin D and neurotransmitters required for the normal growth of osteoblast. DM induced diabetic neuropathy is the com-

monest complication of non-traumatic lower limb amputations in diabetic patients. Although the exact pathogenesis of diabetic neuropathy remains unclear, there are emerging data from *in-vitro* and *in-vivo* clinical studies suggesting that hyperglycemia induced formation of advanced glycation end products (AGEs) may play a key role in the pathogenesis of diabetic neuropathy<sup>[36,37]</sup>. Under hyperglycemic conditions, concentrations of methylglyoxal, 3-deoxyglucosone and glyceraldehyde increase rapidly due to the increased breakdown of glucose. Elevated levels of methylglyoxal, 3-deoxyglucosone and glyceraldehyde lead to the formation of advanced AGEs which in turn modify nerve cell components as well as signal through the receptor for advanced glycation end product (RAGE) expressed on the nerve cells in order to produce different types of cytokines which may have roles on nerve damage<sup>[36-38]</sup>. AGEs have deleterious effect on nerve cells because they modify neuronal proteins including tubulin, neurofilament, laminin and actin through glycation and thereby sequester the nerve function (Figure 2)<sup>[36,37]</sup>.

Beyond the damage of peripheral nerve cells on osteoblast through diabetic neuropathy, DM induced AGEs and angiotensin- II also upregulate the expression of IL-6 that regulates osteoblastic genes required for their survival, differentiation and function<sup>[32,39,40]</sup> (Figure 1).

Reduced vitamin D levels in the body have been identified as a potential risk factor of osteoporosis and bone fractures. Deficiency of Vitamin D in the serum sequesters the intestine to absorb  $Ca^{2+}$  from diet and thereby signals the parathyroid gland to secrete elevated levels of PTH. Hyper secretion of PTH induces bone resorption and inhibit osteoblastogenesis in order to maintain the optimal level of calcium and phosphorus in the blood required for metabolic process and neuromuscular functions<sup>[41,42]</sup>. Through binding of PTH with its receptor PTH-1 expressed on osteoblast triggers intracellular signaling molecules such as PKA, mitogen activated protein kinase A (MAPK), cyclic AMP-responsive element binding protein, AP1 and RUNX2 and thereby induce the expression of the MGP responsive element<sup>[1]</sup> (Figure 1).

Bone marrow derived endothelial progenitor cells (EPCs) may have roles in angiogenesis during bone healing<sup>[3,43]</sup>. DM down regulates the expression of EPCs through different mechanisms and hereby decreases the rate of angiogenesis required for bone formation in the fracture sites<sup>[3,44,45]</sup>. Mesenchymal stem cells (MSC) derived from bone marrow act as a precursor of osteoblast formation<sup>[46-48]</sup>. Several labs based trials have come to the decision that, DM is responsible for the upregulation of peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ), adipocyte fatty acid binding protein (aP2), TNF- $\alpha$  and consequently decrease the availability of MSC for osteoblast formation but increase the availability of MSC for adipocyte formation<sup>[3,4,34,35,48,49]</sup>. So it is intuitive that, in addition to direct interference with osteoblast formation DM also responsible for the deposition of lipid in the bone marrow and thereby leading to the expansion of marrow cavity as well as decreases the rate of blood flows to the

bone which is required for the transfer of nutrients<sup>[3,5]</sup>. The transformation of osteoblast to adipocyte makes the reduction of osteoblast number available for bone formation<sup>[3,50]</sup>. Advanced glycation end products (AGEs) have been identified as a biomarker for the increased risk of fractures because it decreases the synthesis of type I collagen and thereby decreases the bone strength. It is now well researched that DM is responsible for the over expression of AGE and have roles in bone rigidity<sup>[51-53]</sup>.

Several experimental studies implicated that, insulin has an anabolic effect on osteoblast development and it is intuitive that, insulin may exert its effect on osteoblast through IR-GRB2-ERK mediated pathway<sup>[4,54]</sup> (Figure 1). Beyond the synthesis of insulin pancreatic  $\beta$  cells also produce other osteoporotic factors including amylin and preptin. Amylin induces bone formation and sequesters bone resorption, preptin induces osteoblast differentiation and mineralization as well as reducing the apoptosis of osteoblast<sup>[4]</sup>. Osteocalcin is a peptide which positively regulates osteogenesis. DM limits the production of osteocalcin through the negative regulation of osteoblast by decreased synthesis of insulin, amylin and preptin. Testosterone is also an important factor of osteogenesis and it is obvious that, limited production of osteocalcin reduces the production of testosterone from the testes<sup>[4]</sup>.

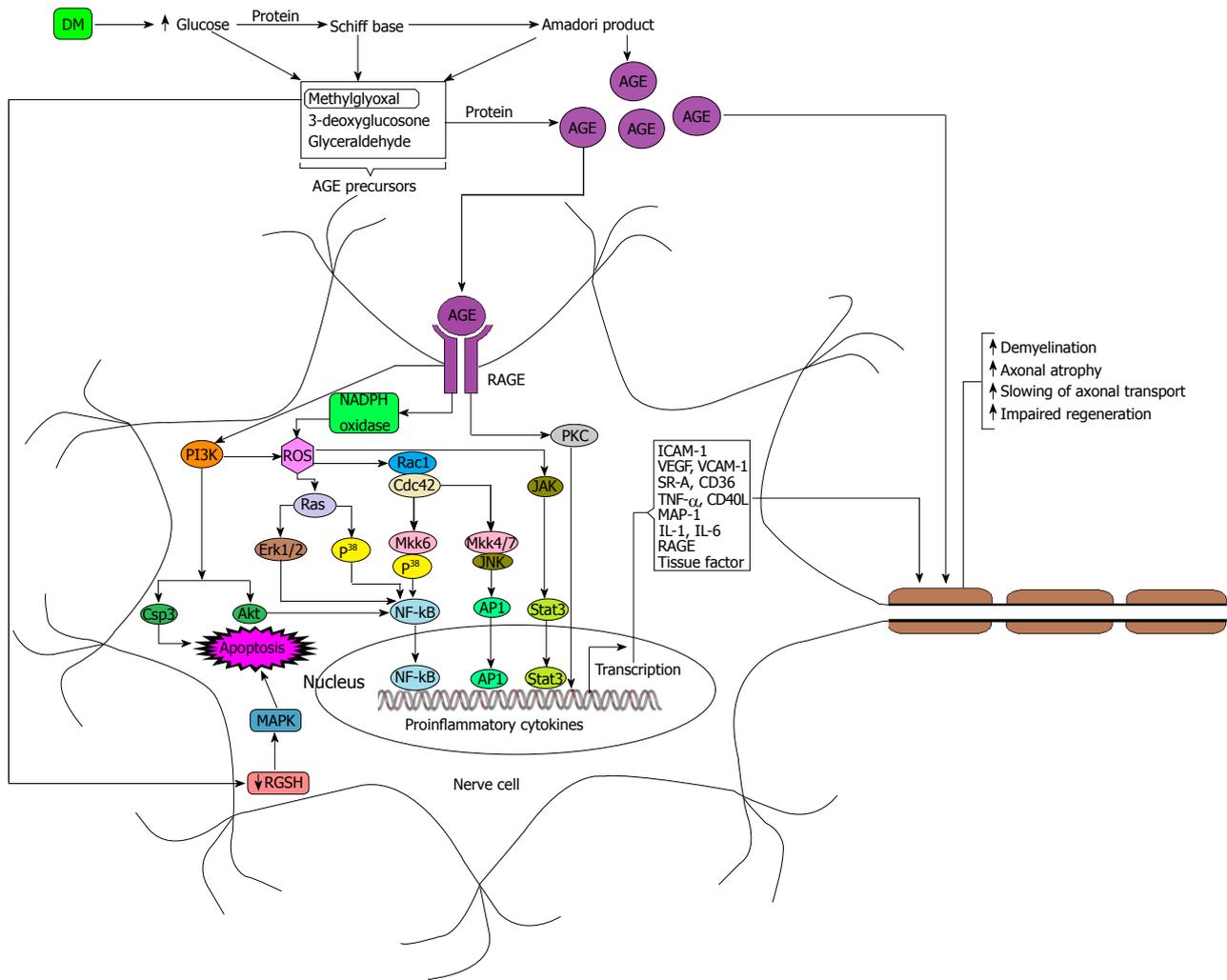
### Osteoclast

Osteoclasts are cells that derived from the monocyte-macrophage cell lineage and strongly participate in osteoclastogenesis. It is well documented that different types of mediators such as nuclear factor  $\kappa$ -B (NF- $\kappa$ B), receptor activator for nuclear factor  $\kappa$ -B ligand (RANKL), osteopontin (OPN), parathyroid hormone (PTH), macrophage colony stimulating factor (M-CSF), and angiotensin- II (AT- II) have prominent roles to induce osteoclastogenesis<sup>[1,13]</sup>.

In general osteoclast exerts its effects in osteoclastogenesis through three possible pathways (1) RANKL mediated; (2) M-CSF mediated; and (3) immunoreceptor tyrosine-based activation motifs (ITAMs). But in inflammatory condition osteoclastogenesis may take place through other pathways like MCP mediated, TNF mediated and IL-6 mediated<sup>[12,55]</sup>.

RANKL is a key factor derived from osteoblast and stromal cells, binds with the receptor expressed on the cell surface of monocyte-macrophage cell lineage and thereby triggers the differentiation of pre osteoclast to osteoclast through activating NF- $\kappa$ B and NFATc1<sup>[56]</sup>. RANKL inhibits the apoptosis of osteoclast through inducing the anti-apoptotic enzyme protein kinase B (PKB) (Figure 3). RANKL also responsible for the production of reactive oxygen species (ROS) including free radicals, oxygen ions and peroxides which are potent inducer of osteoclastogenesis<sup>[1,12,56-59]</sup>.

Binding of RANKL with its receptor RANK activates signal transduction pathways involving the adaptor protein TNF receptor-associated factor 6. Subsequently, several kinases such as p38 MAPK and JUN N-terminal



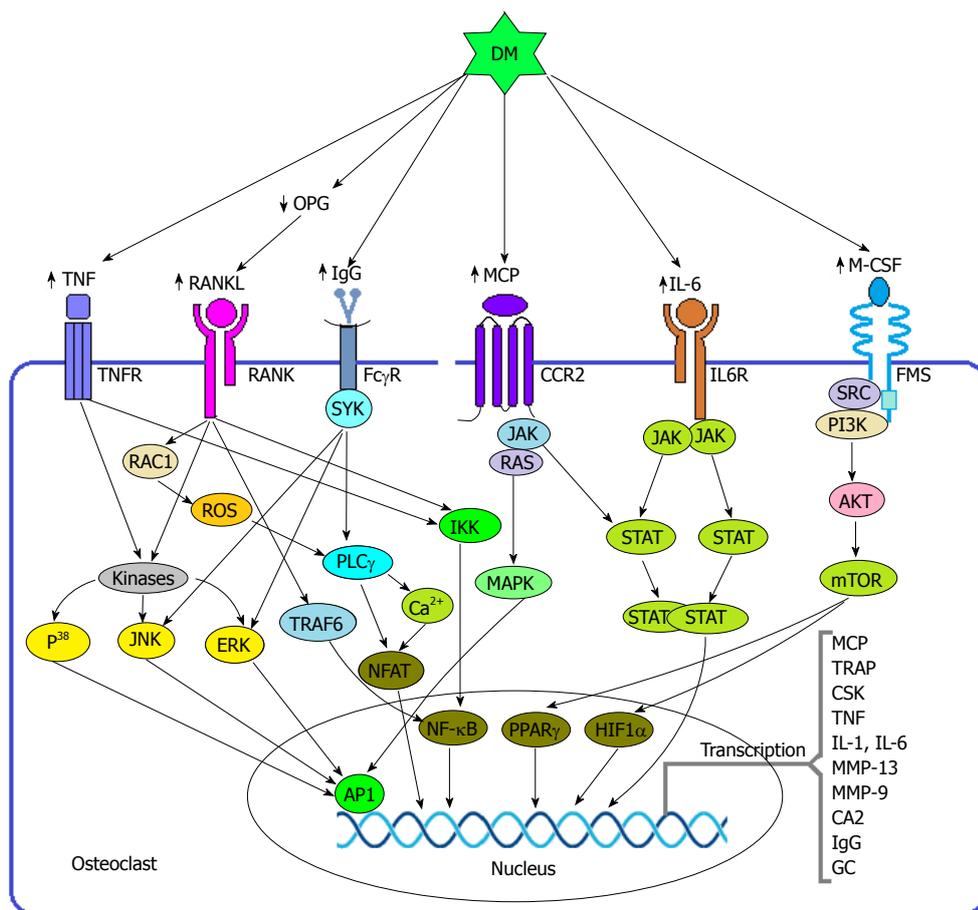
**Figure 2 Diabetes mellitus induced Peripheral nerve damage.** During hyperglycemic condition concentrations of methylglyoxal, 3-deoxyglucosone and glycerlaldehyde increase rapidly due to the increased breakdown of glucose. Elevated levels of methylglyoxal, 3-deoxyglucosone and glycerlaldehyde lead to the formation of advance glycation end products (AGEs) which in turn modify nerve cell components as well as signal through the receptor for advance glycation end product (RAGE) expressed on the nerve cells in order to produce different types of cytokines which may have roles on nerve damage. RAGE induced nicotinamide adenine diphosphate hydrogen (NADPH) oxidase is the major source of reactive oxygen species (ROS) and ROS plays a crucial role to activate nuclear factor kappa B (NF-κB) through Ras-Erk, Rac1-Mkk6 depended pathway. ROS also activates AP-1 and Stat-3 through Rac1-Mkk4/7, JAK-Stat mediated pathway respectively. RAGE may induce apoptosis through PI3K-Csp3 depended pathway as well as activates NF-κB through PI3K-Akt mediated pathway although PI3K may participates in ROS production. Diabetes mellitus (DM) induced methylglyoxal may directly participates in apoptosis through MAPK mediated pathway. Activated NF-κB, AP-1 and Stat3 act congruously to transcribe the genes of proinflammatory cytokines and other factors which are responsible for the destruction of peripheral nerve cells. AGE also participate directly on the modification of axon and thereby reduce the potentiality of signal transduction. DM: Diabetes mellitus; AGE: Advance glycation end product; JAK: Janus kinase; RAGE: Receptor advance glycation end product; ROS: Reactive oxygen species.

kinase 1 are activated, which in turn induce the transcription *via* the various hetero and homodimers of the AP1 family of proteins including FOS, FOSB, FOS-related antigen 1 (FRA1), FRA2, JUN, JUNB and JUND (Figure 3). AP1 regulates the differentiation, proliferation and apoptosis, of various cell types<sup>[12]</sup>.

RANKL is necessary for osteoclastogenesis but an experiment conducted on mouse model showed that, M-CSF acts as a positive catalyst in RANKL activation because the addition of M-CSF requires less time to do a particular resorption process than the RANKL alone<sup>[60]</sup>. Osteoprotegerin (OPG) is a prominent factor for osteoclast activation because the affinity of OPG for RANKL prevents the binding of RANKL with its receptor RANK and thereby decrease the RANKL-RANK medi-

ated pathway of osteoclast multiplication, survival and bone resorption<sup>[1]</sup>.

According to the immunoreceptor tyrosine-based activation motifs (ITAMs), binding of immune complex like immunoglobulin G (IgG) with its receptor FcγR activates spleen tyrosine kinase (SYK), which in turn induces NFATC1 through the activation of phospholipase Cγ (PLCγ) (Figure 3). NFATC1 is an important transcription factor that transcribes the genes that encode calcitonin receptor, tartrate-resistant acid phosphatase, matrix metalloproteinase 13 and cathepsin K. All these factors enable the acidification and degradation of the bony matrix<sup>[12]</sup>. DM is thought to be a potent inducer of IgG because an experiment conducted on mouse model showed that non-obese diabetic mice spontaneously produce natural



**Figure 3 Diabetes mellitus induced regulation of osteoclast.** During normal physiology several osteoblastogenic modulators including RANKL, M-CSF, monocyte chemoattractant protein (MCP), and immunoglobulin G (IgG) binds with their receptors expressed on osteoclast and activates different signal transduction pathway to transcribe the particular gene. Binding of RANKL with RANK triggers several possible pathways to induce the corresponding element. It may induce transcription factor NF-κB through TRAF or IκB kinase (IKK) mediated pathway as well as induces nuclear factor of activated T cells (NFAT) through reactive oxygen species (ROS)-phospholipase Cγ (PLCγ) mediated pathway. RANK also may induce AP-1 through triggering the kinase enzymes. Macrophage colony stimulating factor (M-CSF) activates transcription factors peroxisome proliferator activated receptor γ (PPARγ) and hypoxia inducible factor 1 α (HIF1α) through PI3K-AKT mediated pathway. MCP activates AP-1 signaling through RAS-MAPK mediated pathway which requires the assistance of JAK. IgG also signals through the Fc receptor γ chain (FcγR) to activate NFAT via the induction of PLCγ as well as activates AP-1 through the kinase enzyme systems and both of the pathways require the activation of SYK. During the state of DM, it induces the upregulation of osteoclastogenic factors stated above and thereby induce the differentiation and activity of osteoclast. In addition to the above factors, DM also induces the synthesis of some proinflammatory cytokines which also favor the bone resorption by osteoclast. Interleukin 6 (IL-6) exerts its effect through JAK-STAT mediated pathway although MCP activated JAK may contribute to the activation of STAT to some extent. TNF also activates NF-κB and AP-1 through IKK and Kinase system respectively. CCR2: CC chemokine receptor 2; mTOR: Mammalian target of rapamycin; OPG: Osteoprotegerin; ERK: Extracellular signal regulated kinase; JNK: JUN N terminal kinase; TRAP: Tartrate resistant acid phosphatase; CSK: Cathepsin K; MMP: Matrix metalloproteinase; CA2: Carbonic anhydrase 2; GC: Glucocorticoid.

IgG autoantibodies<sup>[61]</sup>.

Beyond the roles of RANKL and M-CSF in osteoclastogenesis, on the state of hyperglycemia a group of proinflammatory cytokines is activated including TNF, IL-1 and IL-6 and these cytokines have profound effects on the differentiation and activation of osteoclast<sup>[12,62-64]</sup>. Although osteoclast differentiation and activation is primarily dependent on the presence of M-CSF and RANKL, osteoclastogenesis is enhanced in the presence of TNF, IL-1 or IL-6. This is partly a consequence of the induction of RANKL in target cells, but these pro-inflammatory cytokines also responsible for the differentiation and activation of osteoclasts from the preosteoclast. In addition, under normal concentrations of RANKL, TNF can induce the differentiation of monocytes and macrophages to preosteoclasts. The osteoclastogenic ac-

tivity of TNF is mediated by p55 TNF receptor and may be partly counteracted by the activation of the p75 TNF receptor<sup>[12]</sup>.

IL-6 is thought to be the most abundant and effective cytokines in blood because: (1) the concentration of IL-6 and IL-6 receptor (IL-6R) is higher than the other cytokines; (2) IL-6 mediates the production of other cytokines related to osteoclastogenesis like glucocorticoid (Figure 3); and (3) Estrogen deficiency exerts its effects in osteoclastogenesis via IL-6 mediated pathway as well as IL-6 is a potent inducer of IgG production<sup>[12,61,65]</sup>.

DM not only induces the overexpression of RANKL<sup>[3,66]</sup> M-CSF<sup>[3,66]</sup> NF-κB<sup>[67]</sup> and OPN<sup>[34,68]</sup> but also stimulates the over expression of several proinflammatory stimulus such as IL-6, MCP, IgG and TNFs which are so important for the maturation and activation of osteoclast. The

DM may induce monocyte to secrete IL-6 through ROS, PKC, MAPK, and NF- $\kappa$ B mediated pathways<sup>[69,70]</sup>.

Estrogen deficiency stimulates osteoclast formation not only by decreasing the OPG production but also by increasing the production of TNF- $\alpha$ , RANKL and osteoclast precursors through stimulating the T cells<sup>[71,72]</sup>. There is striking evidence on behalf of this regard that, estrogen levels are significantly lower in DM patients<sup>[73]</sup>. Adiponectin is another factor secreted by the adipose tissue and there has been increasing evidence suggest that, adiponectin stimulates the differentiation and mineralization of osteoblast but directly inhibits osteoclast activity and bone resorption<sup>[74]</sup>. Some *in situ* studies have shown that adiponectin percentage is lower in individuals with DM than the individuals without DM<sup>[75]</sup>.

Intracellular ROS mediated oxidative stress plays a crucial role in bone health because ROS promotes RANKL mediated osteoclast differentiation and function. Patients with type 2 DM have shown elevated level of mitochondrial ROS and thus supporting the point that, DM may have another role in ROS mediated osteolysis and bone fractures<sup>[76,77]</sup>. As mentioned before, diabetic neuropathy is a cause of increased production of IL-6, TNF and some other factors, so it is intuitive that, diabetic neuropathy may have a positive role in osteoclast functioning<sup>[12,38,64]</sup>.

## EFFECT OF DIABETES MELLITUS ON MUSCLE CELLS

Muscle atrophy is a physiological condition which associated with the depression of protein synthesis as well as an increase in protein degradation<sup>[78]</sup>. There are some other evidences showed that, DM is associated with diabetic neuropathy mediated muscle atrophy or directly triggers muscle atrophy through TNF- $\alpha$ , NF- $\kappa$ B mediated pathway and thereby induces muscle weakness<sup>[68,79-81]</sup>. Weakness of the muscle is a risk factor of bone fractures because an individual with weak muscles is more likely to fall down than a normal individual. In addition to muscle weakness, diabetic polyneuropathy also induces bone resorption through osteolysis<sup>[82,83]</sup>.

DM is directly associated with muscle atrophy through an increased activity of the ubiquitin proteasome system (UPS) although other pathways may involve in this process<sup>[78]</sup>. There are several inducers of UPS including glucose<sup>[33]</sup> TNF- $\alpha$ <sup>[84,85]</sup> Ang-II<sup>[86]</sup> IL-6, Glucocorticoid (GC)<sup>[85]</sup> and most of them exert their effects on myogenesis responsive gene through NF- $\kappa$ B mediated pathway<sup>[68,78]</sup>.

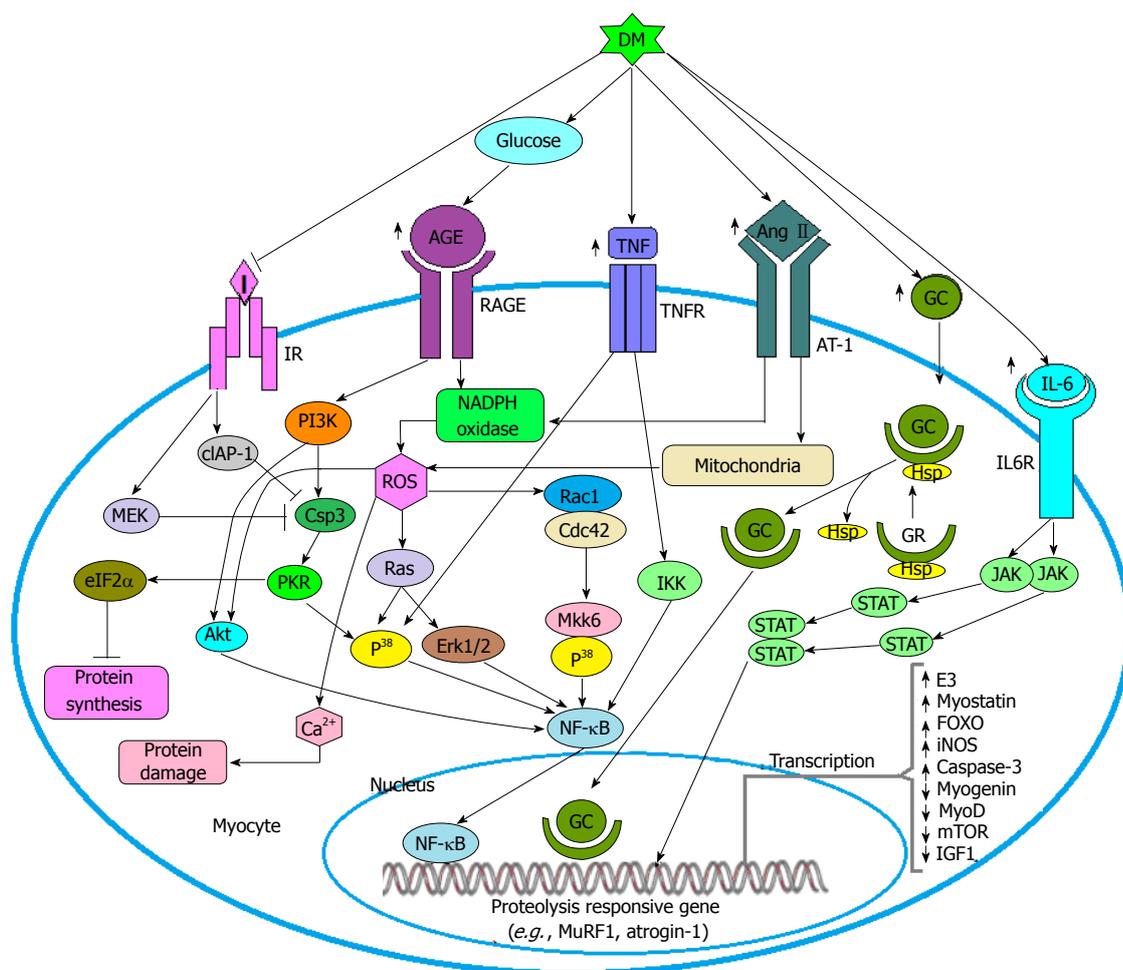
High extracellular glucose concentrations is a potential precursor of AGE formation and several evidences have shown that, AGE may induce the formation of ROS through NADPH oxidase and PI3K/Akt mediated pathway and ultimately activates the transcription factor NF- $\kappa$ B<sup>[37,38,78]</sup>. AGE may induce PKR through caspase-3 mediated pathway and activated PKR then in turn induces NF- $\kappa$ B through P<sup>38</sup> MAPK mediated pathway as well

as activates eIF2 $\alpha$  which would depress protein synthesis by decreasing translational efficiency<sup>[78]</sup>(Figure 4).

Several studies have implicated that TNF- $\alpha$  is a prominent cytokine in cachexia induced muscle atrophy<sup>[84]</sup> as well as a potent inducer of insulin resistance<sup>[87]</sup>. Binding of TNF- $\alpha$  with its receptor expressed on myocyte activates nuclear transcription factor NF- $\kappa$ B through P<sup>38</sup> MAPK or IKK mediated pathway and activated NF- $\kappa$ B then in turn induces the transcription of inducible nitric oxide synthase (iNOS) as well as transcribes the gene MuRF-1 responsible for muscle wasting<sup>[84]</sup>(Figure 4).

Ang-II which is the major peptide of the renin-angiotensin system has been implicated as a modulator of muscle wasting<sup>[88]</sup>. Ang-II exerts its effect on muscle atrophy not only through the generation of ROS but also through the activation of IL-6 and Glucocorticoid as well as through disrupting insulin signaling in muscle cells. It is experimentally determined that, ROS has a significant role in the reduction of muscle strength<sup>[89,90]</sup>. There are two sources of Ang-II induced ROS production (1) NADPH oxidase; and (2) Mitochondria, but NADPH oxidase is thought to be prominent between the two sources. ROS may contribute to muscle wasting activity through three mechanisms (1) by increasing the absorption of Ca<sup>2+</sup> in order to activate calcium-activated proteases; (2) by stimulating the UPS through activating caspase-3; and (3) by up-regulating atrogen-1 and MuRF-1 in muscle to activate the proteasome system through transcribing E3 ligases<sup>[86]</sup>(Figure 4).

Ang-II induced glucocorticoid (GC) plays an important role in muscle wasting because several *in-vivo* and *in-vitro* studies have shown that, addition of different types of GC antagonist of experimental model reduce the rate of muscle wasting<sup>[86,91,92]</sup>. GC exerts its effect on muscle through two ways (1) through sequestering the anabolic action, and (2) through inducing the catabolic action<sup>[91]</sup>. On behalf of the anti-anabolic action firstly, GC inhibits the transport of amino acids into the muscle and thereby limits the protein synthesis<sup>[91]</sup>. Secondly, GC sequesters the stimulatory effects of insulin and insulin like growth factor 1 (IGF-1)<sup>[91,92]</sup>. Thirdly, GC negatively regulates the synthesis of MyoD, an important transcription factor that regulates the differentiation and development of muscle cells as well as required for regeneration and self-renewal of skeletal muscle cells<sup>[92]</sup>. Fourthly, mechanistic target of rapamycin (mTOR) is a kinase protein which regulates the translation of muscle protein. GC inhibits the activity of mTOR through enhancing the transcription of REDD1, a repressor of mTOR function<sup>[91]</sup>. Finally, GC inhibits myogenesis through the downregulation of myogenin, an important a transcription factor required for differentiation of satellite cells into myofibrils<sup>[91,92]</sup>. On behalf of the catabolic activity firstly, GC stimulates the synthesis of several components (*e.g.*, E3) required for UPS through the upregulation of the respective genes including MuRF-1 and atrogen-1<sup>[91,92]</sup>. Secondly, GC induces the overexpression of myostatin a growth regulator which inhibits the development of muscle mass through



**Figure 4 Diabetes mellitus induced regulation of skeletal muscle.** Diabetes mellitus (DM) induced elevated blood glucose is the major source of advanced glycation end product (AGE) which binds with its receptor advanced glycation end product (RAGE) to activate the signal cascade into myocyte. RAGE activation enhances the generation of reactive oxygen species (ROS) through the activation of nicotinamide adenine dinucleotide hydrogen (NADPH) oxidase. Ang-II also induces the production of ROS not only by activating NADPH oxidase but also by inducing the mitochondria. ROS may exert its effects on nuclear factor kappa B (NF-κB) through Rac1-Mkk6 and Ras mediated pathway or accelerate the damage of muscle protein through Ca<sup>2+</sup> dependent pathway. Beyond the generation of ROS, RAGE also activates PI3K which in turn activates NF-κB through Csp3- PKR and Akt mediated pathway. Activated PKR may induce the activation of eIF2α that inhibits protein synthesis. DM induced proinflammatory cytokines interleukin 6 (IL-6) activates the gene through JAK-STAT signaling pathway and TNF activates the factor NF-κB via IKK or MAPK38 induced pathway. Ang- II induced GC also has a role in muscle atrophy and GC exerts its effect through GC-GCR complex mediated pathway. Insulin signaling is also important for muscle growth because it sequesters the activity of Csp3 through inducing the production of cIAP-1 and MEK which are potential inhibitors of Csp3. Type 1 DM reduces the production of insulin and type 2 DM makes the cell insulin resistant, so due to the deficiency of insulin it limits the functioning of cIAP-1 and MEK.

downregulating the proliferation and differentiation of satellite cells<sup>[91,93,94]</sup>. Thirdly, GC induces the breakdown of myofibrillar protein through the upregulation of caspase-3<sup>[89]</sup>. Finally, Forkhead Box O-1 (FOXO-1) is a transcription factor that induces UPS through the upregulation of genes including atrogin-1/MAFbx and MuRF1. Several lab based experiments have come to the decision that, GC induces the production of FOXO-1 through stimulating the respective genes<sup>[91,92]</sup>(Figure 4).

IL-6 is a proinflammatory cytokine which has been implicated as a potential factor of muscle atrophy<sup>[86,95,96]</sup>. Ang-II induced IL-6 upregulates the transcription of serum amyloid A (SAA) and both of the factors (IL-6 and SAA) act synergistically to trigger muscle atrophy<sup>[93]</sup>. An *in-vitro* study has shown that, IL-6 exerts its effect on muscle wasting through JAK/STAT mediated pathway<sup>[97]</sup>(Figure 4).

Insulin deficiency (ID) and insulin resistance (IR) are the hallmark of type-1 and type-2 DM respectively. IR has been implicated as a potential inducer of overall protein degradation as well as caspase-3 mediated actin cleavage. Elevated level of intracellular insulin inhibits caspase-3 protein through MEK and cIAP-1 mediated pathway but during IR or ID condition insufficiency of insulin cannot exert its inhibitory effect on caspase-3<sup>[98]</sup>(Figure 4).

DM induced diabetic retinopathy may be another risk factor of bone fractures because diabetic retinopathy is a leading cause of vision loss and blindness and consequently augments the rate of stumble mediated bone fractures<sup>[99]</sup>. Abnormal movement caused by polyneuropathy and heart failure caused by diabetic cardiovascular complications also promotes the rate of fall<sup>[4,5]</sup>.

Beyond the role of vitamin D in osteolysis, it is intuitively

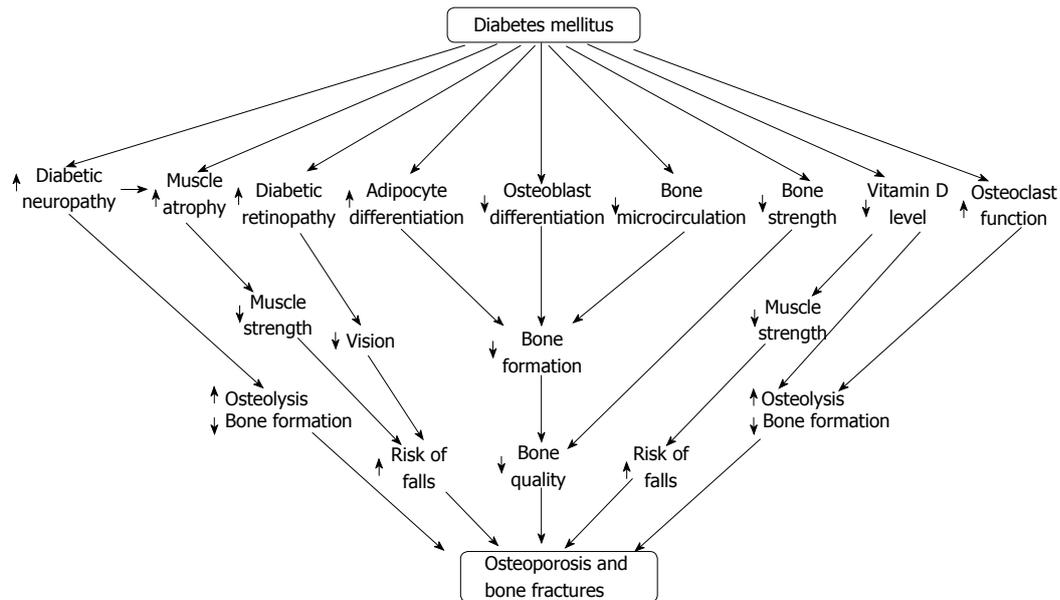


Figure 5 Possible pathways of diabetes mellitus induced osteoporosis.

tive that, vitamin D exerts a range of effects in skeletal muscle cells. Muscle activity specially the power stroke is a  $Ca^{2+}$  depended process and due to the lack of  $Ca^{2+}$  the system will be shut down. An inadequacy of vitamin D can turn down the availability of calcium and phosphorus and thereby postpones the activity of muscles<sup>[100]</sup>. Some *in vitro* and *in vivo* trial have shown that, vitamin D levels are significantly lower in patients with DM<sup>[101,102]</sup>.

## DISCUSSION

Diabetes mellitus may be an obvious cause of osteoporosis and bone fractures due to its broad range of effects on different mediators of the human body. It mainly regulates the bone cells (specifically osteoblast and osteoclast) and the muscles to exert its effects to facilitate osteoporosis as well as reduction of muscle strength<sup>[3,98]</sup>(Figure 5). DM negatively regulates the normal functioning of osteoblast but positively regulates the osteoclast functioning in order to facilitate the process of osteoporosis<sup>[1,98]</sup>. DM reduces the availability of MSC to produce osteoblast but simultaneously increases the availability of MSC for adipocyte formation<sup>[1]</sup>. Due to the continual differentiation and deposition of adipocytes into the bone marrow increase the bone marrow cavity to make the bone fragile as well as decrease the bone microcirculation<sup>[1,99]</sup>. The limitation of osteoblast functioning, over production of adipocytes and fluent functioning of osteoclasts all these effects negatively regulate the bone formation but positively regulate the bone resorption and ultimately cause osteoporosis. DM induced diabetic neuropathy acts as a prominent factor in osteoporosis and muscle atrophy. Diabetic neuropathy inhibits bone formation through sequestering osteoblast formation and function but facilitates osteolysis through inducing the osteoclast generation and function as well as reduc-

ing muscle strength through inducing the expression of different cytokines and ROS<sup>[75,98]</sup>. DM directly causes muscle atrophy through different mediators or indirectly causes muscle atrophy through diabetic neuropathy. DM induced diabetic retinopathy is another risk factor that may cause bone fractures through reducing eye sight<sup>[98]</sup>. Reduced muscle strength as well as reduced eye sight may cause elevated rate of fall down or stumble that may cause bone fractures. Vitamin D is an essential factor of bone and muscle activities because deficiency of vitamin D stimulates the production of PTH which is a negative regulator of osteoblast functioning but a positive regulator of osteoclast functioning which then in turn reduce bone formation and increase bone resorption respectively<sup>[53,54]</sup>. DM induced Vitamin D deficiency also causes the reduction of muscle strength because it lowers the rate of  $Ca^{2+}$  absorption by the intestine and thereby reduces the activity of muscle which may be a risk factor of bone fractures through increasing the rate of fall<sup>[55,98]</sup>.

## CONCLUSION

Diabetes mellitus exerts its diabolical effects on bone, neuron and muscle cells through a broad spectrum of mechanisms. It declines the production of various stimuli required for normal homeostasis of the above cells and accelerates the synthesis of several cytokines and other factors which may directly destroy the target cells or indirectly antagonize the signaling pathways of the stimulus. As human body is a network of different pathways so any imbalance on any part of the pathway may tends the body vulnerable to different threats. DM is the potent source of excess glucose in the blood which is the principal key to create a lot of abnormalities in the body including Osteoporosis and bone fractures, cardiovascular disease, diabetic nephropathy, diabetic neuropathy, Dia-

betic retinopathy and muscle atrophy. Other fatal diseases like HIV and cancer may be linked with hyperglycemia and several investigations have been running to elucidate the mystery of DM induced mechanism. Although several drugs are available to treat osteoporosis, regular physical exercise would be a better way to get rid of from this type of life threatening disease.

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## Genetics of type 2 diabetes

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### Abstract

Type 2 diabetes (T2D) is the result of interaction between environmental factors and a strong hereditary component. We review the heritability of T2D as well as the history of genetic and genomic research in this area. Very few T2D risk genes were identified using candidate gene and linkage-based studies, but the advent of genome-wide association studies has led to the identification of multiple genes, including several that were not previously known to play any role in T2D. Highly replicated genes, for example TCF7L2, KCNQ1 and KCNJ11, are discussed in greater detail. Taken together, the genetic loci discovered to date explain only a small proportion of the observed heritability. We discuss possible explanations for this "missing heritability", including the role of rare variants, gene-environment interactions and epigenetics. The clinical utility of current findings and avenues of future research are also discussed.

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**Key words:** Type 2 diabetes; Genetics; TCF7L2; Genome-wide association studies; Heritability

**Core tip:** We review the history and the current state of knowledge regarding the genetic component of type 2 diabetes risk. Genes like TCF7L2 that have been replicated in multiple studies are discussed in detail. The

significance of these findings is discussed and gaps in our knowledge are identified, as are avenues for future research.

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### INTRODUCTION

Diabetes has been recognized as a distinct disease for over 2000 years<sup>[1]</sup> but it was not until 1935 that Hinshworth established that there were two distinct types of diabetes<sup>[2]</sup>. While both common types of diabetes are characterized by sustained elevations of plasma glucose levels, type 1 diabetes is an autoimmune disease that results in complete loss of the insulin-producing  $\beta$ -cells in the pancreatic islets, while type 2 diabetes (T2D) typically results when insulin secretion from the islets fails to keep pace with increasing insensitivity to the action of circulating insulin on its target tissues (particularly muscle, liver, and fat).

The development of T2D is the result of interaction between environmental factors and a strong hereditary component. Environmental risks factors known to impact the development of T2D include obesity, sedentary lifestyle, small or large birth weight and stress. Other nutritional factors and toxins may also play a role<sup>[3]</sup>. These environmental factors clearly play a major role in the development of diabetes, but they do not impact everyone in the same way. Even with the same environmental exposures, some people are more susceptible to developing diabetes than others, and this increased risk appears to be inherited. But while hereditary factors clearly play a role in the development of diabetes, the actual genetic variants involved in this inherited risk were completely unknown prior to the advent of modern genetic technologies. The advance of human genetic studies in the 1980s finally made it possible to try and identify genetic loci that underlie this hereditary component. Here, we will re-

view the heritability of T2D and the various genetic loci identified to date as contributing to this heritability.

## HERITABILITY OF T2D

Estimates for the heritability of T2DM range from 20%-80% and evidence for heritability comes from a variety of population, family, and twin-based studies<sup>[4,5]</sup>. The lifetime risk of developing T2D is 40% for individuals who have one parent with T2D and 70% if both parents are affected<sup>[6]</sup>. First degree relatives of individuals with T2D are about 3 times more likely to develop the disease than individuals without a positive family history of the disease<sup>[7]</sup>. The concordance rate in monozygotic twins is about 70% whereas the concordance in dizygotic twins has been observed to be only 20%-30%<sup>[8]</sup>. The observed familial risk is higher when studies are restricted to parents in the 35-60 year age range, indicating the greater role played by environmental factors in those who develop diabetes late in life<sup>[9]</sup>. It should be noted that a significant proportion of this heritability reflects heritability of obesity rather than diabetes, obesity being a major driver of T2D in every population.

This familial clustering of T2DM risk found in various family studies is not entirely due to genetic factors. Epigenetic processes can produce inherited risk over one or several generations, intrauterine and pregnancy related factors can impact the risk of siblings, and shared environment can be hard to control for in many such studies. Thus the genetic component of T2D may turn out to be less than what was estimated in older studies.

## GENETIC ARCHITECTURE OF T2DM DISEASE RISK

The detailed genetic architecture of T2D risk has not yet been precisely defined. A relatively small percentage (5% or less) of non-autoimmune diabetes is due to monogenic causes and is classified as monogenic diabetes of the young or MODY (previously referred to as maturity onset diabetes of the young). These cases are understood to be caused by single genes of high penetrance, of which mutations in the Hepatocyte nuclear factor-1A (HNF1A) and the glucokinase (GCK) gene are the most common<sup>[10]</sup>. These forms of diabetes are sometimes misdiagnosed as T2D but clinically they are distinct diseases. They will not be considered further in this review but it should be kept in mind that the boundaries between polygenic and monogenic forms are not always sharply defined at the genetic level. Polymorphisms in genes involved in monogenic forms of diabetes also play a role in polygenic T2D<sup>[11]</sup>.

T2D itself is thought to be a polygenic disorder that develops due to complex interaction between multiple genes and environmental factors. How these genes interact with each other and with the environment to produce T2D is still poorly understood. Unlike T1D, where the genetic risk is mostly concentrated in the HLA region,

the genetic component of T2D risk is not concentrated in one region and appears to be the result of the interaction of multiple genes scattered all across the genome. It is possible; even likely, that the genetic component of T2D is due to multiple common genetic variants of small effect (common disease common variant hypothesis) but this is by no means certain and it may turn out that the effect is due to multiple rare variants or even a few rare variants of large effect<sup>[12-14]</sup>.

## IDENTIFICATION OF DIABETES RISK GENES

### Linkage studies

Linkage is the tendency for genes and other genetic markers to be inherited together because of their location near one another on the same chromosome. While linkage analysis is simple in principle, it has relatively poor resolution as only a few hundred markers were usually genotyped across the genome, and the regions identified by linkage could include millions of base pairs and hundreds of genes. While these methods were quite successful in detecting rare variants of large effect (*e.g.*, classical single gene disorders), they proved relatively unsuccessful in identifying genes that are involved in complex polygenic disorders. These studies only revealed two genes, calpain 10 (*CAPN10*) and transcription factor 7-like 2 (T-cell specific, HMG-box) (*TCF7L2*) that were reliably identified as being associated with T2D.

**CAPN10:** *CAPN10* encodes a cysteine protease that is part of the calpain family, a large family of ubiquitously expressed genes that play multiple roles in intracellular remodeling, post-receptor signaling and other intracellular functions. It became the first T2D gene to be discovered by linkage analysis when a locus on chromosome 2 was associated with T2D in 1996<sup>[15]</sup>. Initially the locus was labeled NIDDM1 but the gene (or genes) involved were not identified. In 2000 the causative gene was finally identified as *CAPN10*<sup>[16]</sup>. Subsequent studies did not always confirm this finding but larger meta-analyses have shown that variants in *CAPN10* are likely to be truly associated with T2D<sup>[17]</sup>. At this time the function of this gene in glucose metabolism remains unknown and its link to T2D, while confirmed in several populations, is not always consistent<sup>[18-20]</sup>.

**TCF7L2:** *TCF7L2* was discovered as a T2D susceptibility gene after a strong linkage signal was mapped to chromosome 10q in a Mexican-American population<sup>[21]</sup>. This region was later fine-mapped in the Icelandic population and confirmed in United States and Danish cohorts, where the risk locus was found to be located in intron 3 of the *TCF7L2* gene<sup>[22]</sup>. The association between T2D and a number of single-nucleotide polymorphisms (SNPs) in the *TCF7L2* gene has since been strongly confirmed in multiple Genome-wide association studies (GWAS) in different ethnic groups and this gene remains the most

replicated and most strongly associated T2D risk gene at this time<sup>[23]</sup>. We will discuss this gene further in the GWAS section of this review.

### Candidate gene studies

In candidate gene studies, genes already suspected of playing a role in the pathogenesis of T2D were studied through focused sequencing efforts. The usual strategy was to focus on genes already known to be involved in glucose metabolism, insulin secretion, insulin receptors, post-receptor signaling and lipid metabolism. Somewhat to the surprise of investigators, most of the genes known to be involved in insulin secretion and action were not found to be associated with T2D in the population. The relatively few genes that were found to be associated with T2D include peroxisome proliferator-activated receptor gamma (*PPARG*), insulin receptor substrate 1 (*IRS1*) and *IRS-2*, potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*), Wolfram syndrome 1 (wolframin) (*WFS1*), HNF1 homeobox A (*HNF1A*), HNF1 homeobox B (*HNF1B*) and *HNF4A*. Other genes including *RAPGEF1* and *TP53* were identified using an algorithm that prioritizes candidate genes for complex human traits based on trait-relevant functional annotation but have not been consistently replicated in later studies<sup>[24]</sup>.

***PPARG*:** *PPARG* gene was an attractive candidate gene for T2D because it encodes the molecular target of thiazolidinediones, a commonly used class of anti-diabetic medications. It was found that a proline to arginine change at position 12 in the *PPARG* gene led to a 20% increase in the risk of diabetes. This finding has since been confirmed in some other populations and other polymorphisms in this gene have been found to play a role in some cases of diabetes<sup>[25]</sup>. Even so, the significance of these mutations was not replicated in all populations and the contribution of these polymorphisms to the worldwide prevalence of diabetes remains low<sup>[26,27]</sup>.

***IRS1* and *IRS-2*:** Insulin receptor substrate *IRS-1* and *IRS-2* genes encode peptides that play an important role in insulin signal transduction. Polymorphisms in these genes were found to be associated with decreased insulin sensitivity in some populations<sup>[28,29]</sup> but as with other candidate genes, the role played by these polymorphisms in the global burden of diabetes and related insulin-resistance disorders like PCOS remains small.

***KCNJ11*:** *KCNJ11* gene encodes the Kir6.2 ATP-sensitive potassium channel that plays an important role in the regulation of insulin secretion by beta cells. Activating mutations in this gene are a well-established cause of neonatal diabetes. A missense polymorphism in *KCNJ11* was found to be associated with T2D and confirmed in subsequent studies<sup>[30]</sup>. The odds ratio of developing T2D is about 1.2 in carriers of the risk allele and this allele was also found to be associated with decreased insulin secretion in different populations<sup>[31-33]</sup>.

***WFS-1*:** *WFS-1* gene encodes Wolframin, a protein that is defective in individuals suffering from the Wolfram syndrome (characterized by diabetes insipidus, juvenile diabetes, optic atrophy, and deafness). *WFS1* gene appears to be involved in beta cell function and 2 SNPs in *WFS-1* were found to be significantly associated with T2D in a large case-control study involving about 24000 samples<sup>[34]</sup>. This was subsequently confirmed in other studies in different populations<sup>[35]</sup>. These studies provided evidence that beta cell dysfunction plays a critical role in the development of T2D and pointed out novel genes that play a previously unknown role in beta cell survival and function, but their role in the global burden of diabetes remains minor.

***HNF1A*, *HNF1B* and *HNF4A*:** *HNF1A*, *HNF1B* and *HNF4A* are all known MODY genes (*i.e.*, genes that harbor rare high penetrance mutations that cause monogenic diabetes of the young). These genes play a role in the development of the liver, in the regulation of hepatic metabolic functions, and in the development and functioning of beta cells. Variants in these genes that do not lead to MODY have been found to be associated with decreased insulin secretion and an increase in the risk of T2D in various populations, but as with other candidate genes, their role in worldwide diabetes prevalence appears to be relatively small<sup>[36-38]</sup>.

### Genome wide association studies

Candidate gene studies and linkage analysis identified a few T2D risk genes, but their overall contribution to the observed heritability of T2D remained small and it was clear that other techniques were needed to look for variants that were not easily identified by these methods. With the development of high-throughput SNP genotyping technology and the availability of Hapmap data, it became possible to scan hundreds of thousands of SNPs that were in linkage disequilibrium with millions of SNPs across the genome. *TCF7L2*, already identified *via* linkage studies, was the most significant and most replicated signal found in GWAS studies, but these studies also helped to identify scores of other genetic loci that appear to be linked to T2D<sup>[39]</sup>. Over the last 6 years, the number of known T2D variants has risen to over 60; including confirmation of variants identified earlier by candidate gene and linkage studies. While most studies have focused on European populations, this is being rectified as more studies of Asian, African and other populations become available.

Since obesity is a major contributor to the development of T2D, genes that increase the risk of obesity also show up in GWAS for T2D. These include some frequently replicated genes include like *FTO* and *MC4R*; these genes seem to primarily impact obesity risk and effect T2D risk mostly *via* their effect on obesity (though *FTO* may have a small but detectable influence on T2D risk independent of the risk of obesity). Here we will focus on genes that specifically increase the risk of T2D,

independent of obesity. The most important of these include

**TCF7L2:** This remains the most significant and consistently replicated gene linked to T2D. It was initially discovered by linkage studies, then confirmed in the very first large-scale GWAS study conducted in a French population by Sladek *et al*<sup>[40]</sup>. This publication was followed in quick succession by several other major GWAS paper, including the landmark Wellcome Trust study that genotyped 2000 individuals with T2D along with 3000 controls and found that TCF7L2 was the most robust T2D signal, with an odds ratio of 1.36 for carriers heterozygous for the risk allele<sup>[41]</sup>. This finding was then replicated in almost every human population studied<sup>[42-48]</sup> and remains the most robust T2D risk gene identified to date. Carriers of the various identified risk alleles have an OR of 1.4<sup>[49]</sup> and homozygotes may have an OR of 2.5.

TCF7L2 encodes a transcription factor that is a member of the Wnt signaling pathway and is known to be active in the beta cells. Studies in multiple ethnicities indicate that the risk allele is present in intron 3 of the *TCF7L2* gene. An early investigation by Lyssenko *et al*<sup>[50]</sup> revealed that the risk alleles increased the level of TCF7L2 protein in beta cells and was associated with impaired insulin secretion, incretin effects and enhanced rate of hepatic glucose production. *TCF7L2* expression in human islets was increased 5-fold in T2D, particularly in homozygotes and overexpression of *TCF7L2* in human islets reduced glucose-stimulated insulin secretion. These findings were replicated in several subsequent studies, indicating that TCF7L2 probably plays a role in causation of T2D by decreasing insulin secretion from beta cells, perhaps by altering the action of incretins that modulate the insulin response to meals<sup>[51,52]</sup>. Other studies indicate that alternative splicing of this gene can lead to the production of different isoforms in different tissues and the presence of specific isoforms in adipose tissue may be related to insulin sensitivity in that tissue<sup>[53,54]</sup>. It is also possible that T2D risk is conferred by multiple mechanisms, including decreased beta cell insulin response and decreased insulin sensitivity in target tissues like adipose tissue. A recent murine study shows that, at least in mice, when *TCF7L2* is knocked out in liver cells it leads to hypoglycemia and when it is overexpressed it causes hyperglycemia, but there is no effect when it is knocked out in the beta cells<sup>[55]</sup>. This indicates that the liver may also be an important site where *TCF7L2* variants influence glucose metabolism. Finally, there are indications that this gene may play a role in cancer as well as in diabetes<sup>[56,57]</sup>. Thus, the discovery of its association with diabetes has opened up several new avenues of research and should eventually lead to the characterization of previously unknown physiological mechanisms that play a role in both diabetes and cancer.

**HHEX:** hematopoietically expressed homeobox (*HHEX*) While TCF7L2 remains the strongest T2D signal in

GWAS studies from across the globe, several other genes have been repeatedly identified in different populations as being associated with T2D. *HHEX* was identified as one such gene in multiple studies in both Caucasian and Asian populations<sup>[58]</sup>. Located on chromosome 10q, this gene is also a member of the homeobox family and encodes a transcription factor involved in Wnt signaling. Risk alleles appear to confer an OR of developing T2D of 1.5. The mechanism by which this gene confers diabetes risk remains poorly understood.

**SLC30A8:** Solute carrier family 30 (zinc transporter), member 8 (*SLC30A8*). This gene encodes for a protein that is involved in the storage and secretion of insulin granules and that is expressed at a high level only in the pancreas, particularly in the islets of Langerhans<sup>[59]</sup>. This provides an obvious mechanism by which it may be involved in conferring T2D risk and this association has been replicated in multiple studies in different populations<sup>[60-62]</sup>. Interestingly, this gene has also been found to be associated with the development and progression of type 1 diabetes<sup>[63]</sup> though this has not been confirmed in all studies<sup>[64]</sup>.

**CDKN2A/B:** Cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*). SNPs located upstream of the *CDKN2A2B* genes have been associated with the risk of T2D in multiple large GWAS. These genes are located on chromosome 9p21 and generate several transcript variants. At least three alternatively spliced variants of *CDKN2A* encoding distinct proteins have been reported, two of which are known to function as inhibitors of *CDK4* kinase. *CDKN2B* is also located in the same region and generates at least 2 splice variants. Both genes are important cell cycle regulators with a role in tumor suppression. This region was found to be associated with T2D in multiple GWAS studies in different populations and it is estimated that the risk alleles confer an odds ratio for development of T2D of between 1.2 and 1.5<sup>[65]</sup>. How variations in these genes alter diabetes risk remains unclear but recent research points to a role in insulin secretion rather than insulin action<sup>[66]</sup>. These variants also show up in GWAS for cardiovascular disease, in particular for atherosclerosis, but the mechanism underlying this association remains unknown<sup>[67]</sup>.

**IGF2BP2:** insulin-like growth factor 2 mRNA binding protein 2 (*IGF2BP2*) encodes a protein that binds to the 5' UTR of the insulin-like growth factor 2 (IGF2) mRNA and thereby regulates IGF2 translation. Alternate transcriptional splice variants, encoding different isoforms, have been characterized<sup>[68]</sup>. This gene has been found to be associated with T2D risk in multiple GWAS<sup>[69,70]</sup>. As with other variants like *HHEX* and *CDKN2A/B*, it may play a role in beta cell function<sup>[65]</sup> but the mechanism by which it influences T2D risk remains largely unknown.

Other genes linked to T2D risk include *CDKAL1* (CDK5 regulatory subunit associated protein 1-like 1),

**Table 1** Thirty-eight genetic variants associated with type 2 diabetes at genome-wide significance

Locus	Chr	Risk allele frequency	OR (95%CI)
NOTCH2	1	0.11	1.13 (1.08-1.17)
PROX1	1	0.5	1.07 (1.05-1.09)
IRS1	2	0.61	1.19 (1.13-1.25)
THADA	2	0.92	1.15 (1.10-1.20)
RBMS1/ITGB6	2	0.57	1.11 (1.08-1.16)
BCL11A	2	0.46	1.08 (1.06-1.10)
GCKR	2	0.62	1.06 (1.04-1.08)
IGF2BP2	3	0.29	1.17 (1.10-1.25)
PPARG	3	0.92	1.14 (1.08-1.20)
ADCY5	3	0.78	1.12 (1.09-1.15)
ADAMTS9	3	0.81	1.09 (1.06-1.12)
WFS1	4	0.27	1.13 (1.07-1.18)
ZBED3	5	0.26	1.08 (1.06-1.11)
CDKAL1	6	0.31	1.12 (1.08-1.16)
JAZF1	7	0.52	1.10 (1.07-1.13)
GCK	7	0.2	1.07 (1.05-1.10)
KLF14	7	0.55	1.07 (1.05-1.10)
DGKB/TMEM195	7	0.47	1.06 (1.04-1.08)
SLC30A8	8	0.75	1.12 (1.07-1.16)
TP53INP1	8	0.48	1.06 (1.04-1.09)
CDKN2A/B	9	0.79	1.20 (1.14-1.25)
TLE4	9	0.93	1.11 (1.07-1.15)
TCF7L2	10	0.25	1.37 (1.28-1.47)
HHEX	10	0.56	1.13 (1.08-1.17)
CDC123/CAMK1D	10	0.23	1.11 (1.07-1.14)
KCNQ1	11	0.61	1.40 (1.34-1.47)
KCNJ11/ABCC8	11	0.5	1.15 (1.09-1.21)
CENTD2	11	0.88	1.14 (1.11-1.17)
MTNR1B	11	0.3	1.09 (1.06-1.12)
KCNQ1	11	0.52	1.08 (1.06-1.10)
HMG2	12	0.1	1.10 (1.07-1.14)
TSPAN8/LGR5	12	0.23	1.09 (1.06-1.12)
OASL/HNF1A	12	0.85	1.07 (1.05-1.10)
PRC1	15	0.22	1.07 (1.05-1.09)
ZFAND6	15	0.56	1.06 (1.04-1.08)
FTO	16	0.45	1.15 (1.09-1.22)
HNF1B	17	0.43	1.12 (1.07-1.18)
DUSP9	X	0.12	1.27 (1.18-1.37)

Modified from Florez *et al*<sup>[71]</sup>.

*HMG2* (high mobility group AT-hook 2), *KCNQ11* (potassium voltage gated channel, KQT like subfamily, member 1) and *NOTCH2-ADAM30* (Notch 2-ADAM metallopeptidase domain 30). Their exact role in the pathophysiology of T2D remains mostly unknown. A list of these and other variants is given below in Table 1.

As can be seen in Table 1, the odds ratios for individual risk alleles are generally less than 1.3 (*TCF7L2* and *KCNQ1* being the most prominent exceptions) and it has been estimated that all the risk alleles identified to date can only explain about 10% of the observed heritability of T2D. Thus these alleles cannot be used to estimate the genetic risk of developing T2D in an individual patient with any degree of certainty since a simple family history will be much more informative than a detailed genotype at this point. But the discovery of these genes has opened entirely new avenues in our quest to understand the regulation of glucose metabolism and the development of T2D. For example, prior to these genetic

studies, no one could have predicted that *TCF7L2* plays any role in glucose regulation. But initially *via* linkage studies, and then in multiple GWAS, it has been shown to be the single most significantly associated diabetes risk gene in the world. This has led to intensive investigation of its physiological role and though those investigations are at an early stage, it is hoped that they will eventually yield a new and more complete understanding of the mechanisms that regulate insulin secretion and action and whose alteration may lead to an increased risk for T2D. That in turn may lead to the identification of new drug targets, diagnostic tests, and targeted therapies (pharmacogenomics).

### What do these genes do?

The fact that many of these genes are active in beta cells or may be involved in insulin secretion support the notion that beta cell dysfunction is a crucial final step on the path to diabetes<sup>[72,73]</sup>. Very few of these genes seem to play a role in insulin sensitivity (though that may change as more information becomes available) and genes involved in the insulin signaling pathway rarely show up in T2D GWAS studies. When indices of beta-cell function (*HOMA-B*) and insulin sensitivity (*HOMA-IR*) derived from paired fasting glucose and insulin measures from 37000 individuals were used to try and identify the function most affected by various T2D risk genes, it was found that risk alleles at ten loci (*MTNR1B*, *SLC30A8*, *THADA*, *TCF7L2*, *KCNQ1*, *CAMK1D*, *CDKAL1*, *IGF2BP2*, *HNF1B* and *CENTD2*) were associated ( $P < 0.05$ ) with reduced beta-cell function, and only three loci (*PPARG*, *FTO* and *KLF14*) were associated with reduced insulin sensitivity<sup>[74]</sup>.

It is possible that this may be because rare variants have a greater impact on insulin sensitivity or because environmental factors play a greater role in altering insulin sensitivity and thus swamp underlying genetic variation in risk. Still, this finding was not expected when candidate gene studies were initiated and shows how agnostic high throughput methods like GWAS can help to generate novel hypotheses and illuminate new aspects of biology. Some of the genes found to be associated with T2D also appear to be linked to dyslipidemia, atherosclerotic heart disease and cancer and it is possible that as we learn more about the role of these genes, we may be able to understand more about the relationship between T2D and other components of the metabolic syndrome as well as cancer<sup>[71]</sup>.

**Gene-environment interactions:** It is abundantly clear that the risk of developing T2D is heavily influenced by environmental factors. Since our genetic code does not change significantly in one or two generations, the recent secular trend in diabetes must be due mostly to changes in the environment. Increased adiposity is the single most significant factor in the development of T2D and the epidemics of obesity and T2D largely parallel one another. The increasing prevalence of obesity is thought

to be related primarily to changes in dietary habits and our increasingly sedentary lifestyle, though other factors (including toxins and infectious agents) may play a role. Genes may influence the risk of diabetes not only by directly altering insulin action or secretion, but also by altering how any given individual interacts with these environmental factors. Even within the same broad environment, individuals vary greatly in their adoption of unhealthy lifestyles and their willingness to change such lifestyles. By influencing who adopts a more unhealthy diet (this includes genetic influence on taste and food preferences), who exhibits greater willingness to change unhealthy behaviors<sup>[75]</sup>, who burns more calories at rest, who exhibits greater activity levels when not actively exercising, what kind of microbiome an individual carries, and who opts for a more sedentary lifestyle, genetic factors can play a role in determining who becomes obese or develops diabetes in any given environment<sup>[76]</sup>. These gene-environment interactions may be extremely complex and may be one reason why such a small proportion of the heritability of T2D has been explained at this time<sup>[77]</sup>.

### Epigenetics

Epigenetics refers to heritable changes in gene function that occur without a change in nucleotide sequence. Mechanisms like DNA-methylation, histone acetylation and non-coding RNAs are used by the cell to regulate gene expression in response to environmental cues and can persist for an individual's lifetime and can be passed on over 2-3 generations<sup>[78]</sup>. It is well known that the maternal environment and early infancy can alter the lifelong risk of chronic diseases. For example, infants who are born small for gestational age are at an increased risk for the development of obesity and T2D as adults. Some or most of this risk may be due to epigenetic changes in critical genes and animal experiments<sup>[79]</sup> and initial human studies suggest that such mechanisms may indeed explain the impact of intrauterine nutrition and birth weight of future risk of diabetes, obesity and metabolic syndrome<sup>[80]</sup>. It is thus possible that some of the observed heritability of T2D is due to epigenetic changes during intra-uterine life that are the result of maternal environmental influences, rather than inherited variations in the DNA sequence. As our understanding of epigenetics advances and as the ability to profile genome-wide DNA methylation and other epigenetic mechanisms becomes more widely used, we are likely to see important discoveries regarding the epigenetic changes that alter the risk of T2D. Epigenetic profiling may also help to identify novel genes that play a role in the pathogenesis of T2D just as GWAS led to the identification of multiple genes that were previously unsuspected of having a role in diabetes.

### Risk prediction based on genetic information

While we know that a person's future risk of developing T2D has a significant heritable component and believe that most of this inherited risk is associated with particu-

lar genotypic features (in most cases, multiple variants of small effect?), and have identified several risk variants in genome-wide association studies, these variants still explain a relatively small proportion of the observed heritability. Several studies have found that a risk score based on traditional risk factors (BMI, family history, age, sex, HDL, triglycerides, *etc.*) consistently outperforms any set of genetic markers and the addition of known genetic markers does not significantly improve prediction based on traditional risk factors<sup>[81-83]</sup>.

This indicates that our current state of knowledge regarding specific genetic markers is still incomplete and fails to explain most of the inherited risk. But as more data becomes available and better statistical techniques are applied to analyze gene-gene and gene-environment interactions, this predictive ability is likely to improve<sup>[84]</sup>. Even before that happens, these genetic discoveries have already provided important new insights into the pathophysiology of T2D and as the physiologic role of these genes in glucose regulation becomes clearer, these discoveries can be expected to lead to better diagnostic and therapeutic tools. Potential applications are not limited to better risk prediction, new drug targets and better targeted drug therapy; some time in the future when our technologies have improved far beyond current levels, they may include the ability to alter the risk of diabetes using gene-therapy or epigenetic reprogramming.

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## Diabetic nephropathy: Treatment with phosphodiesterase type 5 inhibitors

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### Abstract

The importance of nitric oxide (NO) in vascular physiology is irrefutable; it stimulates the intracellular production of cyclic guanosine monophosphate (cGMP), initiating vascular smooth muscle relaxation. This biochemical process increases the diameter of small arteries, regulating blood flow distribution between arterioles and the microvasculature. The kidney is no exception, since NO predominantly dilates the glomerular afferent arterioles. It is now evident that the vascular production of cGMP can be augmented by inhibitors of phosphodiesterase type 5 (PDE 5), the enzyme which breakdowns this cyclic nucleotide. This has clinical relevance, since diabetic nephropathy (DN) a major microvascular complication of diabetes mellitus and the most common cause of end-stage renal disease, increases intraglomerular capillary pressure, leading to glomerular hypertension. PDE 5 inhibitors may have, therefore, the potential to reduce glomerular hypertension. This review describes the use of PDE 5 inhibitors to improve the metabolic, haemodynamic and inflammatory pathways/responses, all of which are dysfunctional in DN.

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**Key words:** Diabetic nephropathy; Phosphodiesterase type 5; Glomerular filtration rate; Inflammation; Angiotensin II

**Core tip:** Diabetic nephropathy a leading cause of end-stage renal disease, is characterized by dysfunctional metabolic, haemodynamic and inflammatory pathways leading to glomerular hypertension. These pathways were normalized following treatment with phosphodiesterase type 5 inhibitors, which initiated renal vascular smooth muscle relaxation. This therapeutic option for treating diabetic nephropathy may negate the need for costly renal dialysis or transplantation.

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### INTRODUCTION

Chronic kidney disease is increasing worldwide at an annual rate of 8%, with the prevalence higher in developing countries<sup>[1]</sup>. Diabetic nephropathy (DN) is a common underlying cause<sup>[1]</sup>; indeed in many countries it is the main cause of end-stage renal disease (ESRD) and is associated with a high morbidity and mortality<sup>[2-5]</sup>. DN develops due to a complex interaction between metabolic and haemodynamic pathophysiological factors, which lead to renal damage<sup>[6,7]</sup>. It can affect 20%-30% of the diabetic population, who present with an increase in urinary albumin excretion (microalbuminuria) in the earliest stage<sup>[4]</sup>. This may progress to macroalbuminuria and later renal insufficiency and ESRD. There is also evidence of an increase in systemic and vascular markers of inflammation<sup>[8,9]</sup> as the size of the kidney progressively increases<sup>[10]</sup>. Accompanying these changes are abnormalities in the blood biochemical indices of renal function, which precede renal failure<sup>[11]</sup>.

Treatment of DN has focused on the integrated control of dyslipidaemia, glycaemia and blood pressure to reduce microalbuminuria<sup>[3,12-14]</sup>. Nevertheless, although, current treatment strategies may slow the progression of DN to ESRD, it does not fully arrest this process<sup>[9,15]</sup>. Inevitably, some patients require renal replacement therapy (RRT), at an average cost of €40000-50000 per patient per year<sup>[5]</sup>. Not surprisingly, the increasing number of diabetic patients on RRT<sup>[9,16]</sup>, is placing a financial strain on health care systems worldwide<sup>[2,17]</sup>. It has also been established that the cardiovascular morbidity and mortality is higher in diabetic ESRD patients on dialysis compared to those without diabetes<sup>[9]</sup>. Slowing the decline of renal function, from DN to ESRD is of paramount importance and hence there is a clear need for new strategies to treat DN.

One feasible option for treating the DN-induced enhancement of intraglomerular pressure<sup>[7]</sup> and the resultant glomerular hypertension<sup>[18]</sup> is to alter the renal cyclic guanosine monophosphate (cGMP)-NO pathway. This is because NO dilates vessels in the kidney, including the glomerular afferents<sup>[19]</sup>, by stimulating intracellular production of cGMP<sup>[20,21]</sup>, which in turn initiates renal vascular smooth muscle relaxation. It is now evident that the vascular cGMP-NO pathway can be augmented by inhibitors of phosphodiesterase type 5 (PDE 5), the enzyme which breakdowns cGMP<sup>[22]</sup>. These inhibitors (sildenafil, tadalafil and vardenafil) allow cGMP to accumulate and are increasingly used to treat penile erectile dysfunction<sup>[23-25]</sup>, as they cause a significant relaxation of the corpus cavernosum<sup>[24,26]</sup> leading to erection. They may also have a therapeutic role in DN, since PDE 5 expression/activity is abundant in the kidney<sup>[27]</sup> and may contribute towards glomerular hypertension. In this scenario selective inhibition of PDE 5 enzymatic activity would provide renoprotection.

This review describes the use of PDE 5 inhibitors to improve metabolic and haemodynamic pathophysiological factors, as well as inflammatory pathways, all of which are dysfunctional in DN.

## METABOLIC CHANGES

Strict glycaemic control is desirable for DN patients, especially as polyol and hexosamine pathways, the accumulation of [advanced glycation end products (AGEs) formed by glycosylation of proteins, lipids and nucleic acid] and activation of protein kinase C are all thought to play a role in disease progression<sup>[7]</sup>. However, there is little evidence that PDE 5 inhibition reduces diabetic hyperglycaemia, since sildenafil had no effect in streptozotocin-induced diabetic rats<sup>[28]</sup> or in a model of non-insulin-dependent diabetes mellitus, the Otsuka Long-Evans Tokushima Fatty (OLETF) rats<sup>[29]</sup>. Even so, the effect of PDE 5 inhibition on other renal metabolic abnormalities needs to be considered.

### Kidney weight, histology and electron microscopy

Kidney size and weight are typically increased in dia-

betes mellitus, primarily due to glomerular and tubular hypertrophy. An increase in the number of glomerular cells (mainly mesangial and endothelial), in extracellular matrix and in capillary number and size all contribute to this hypertrophy<sup>[30]</sup>. This increase is also evident when kidney weight is corrected for body surface area<sup>[31]</sup>, or in diabetic rats, when expressed as kidney: body-weight, a ratio lowered following treatment with sildenafil<sup>[28]</sup>. In terms of renal histology, glomerular lesions characterized by hypertrophy, mesangial matrix expansion and sclerotic lesions were evident in the OLETF rat kidney and were significantly reduced by sildenafil<sup>[29]</sup>, demonstrating drug-induced amelioration of NIDDM nephropathy.

The reduction in kidney weight and improved histology afforded by treatment with sildenafil, suggests that PDE 5 inhibition may prove to be an important and effective therapeutic option for DN-induced hypertrophy. This is supported by the finding that renal morphological changes induced in spontaneously hypertensive rats (SHR) by cyclosporin A, a potent nephrotoxic immunosuppressant were also improved with a PDE 5 inhibitor (FR226807, Fujisawa Pharmaceutical, Japan)<sup>[32]</sup>.

The earliest ultrastructural abnormality in DN relates to the diffuse thickening of the glomerular basement membrane, which increases as the disease advances. As previously mentioned, several biochemical changes contribute to this process, notably an increase in collagen type IV deposition and impairment of excess extracellular matrix degradation, mesangial expansion by extracellular matrix deposition and increased mesangial cellularity. There are also changes in glomerular epithelial cells (podocytes), including a decrease in number and/or density, with a reduced podocyte per glomerulus ratio, podocyte foot process broadening and effacement, glomerulosclerosis and tubulointerstitial fibrosis<sup>[18,33]</sup>. It would be interesting to establish whether PDE 5 inhibitors can prevent or reverse these DN-induced ultrastructural changes observed under electron microscopy.

### Serum creatinine

Creatinine is a by-product of muscle-derived creatine. In the early stages of DN, kidney compensatory mechanisms maintain serum creatinine levels, but as the disease progresses this compensation fails due to the marked and continuous damage to functioning nephrons. It seems, therefore, that the increased serum creatinine seen in DN indicates the severity of the clinical renal damage. Interestingly, in the first study to treat DN with a PDE 5 inhibitor (vardenafil given orally for one month to alloxan-induced diabetic rabbits) the elevated serum creatinine level was restored to normal<sup>[34]</sup>.

The notion that PDE 5 inhibition can ameliorate the progression of renal damage has been examined in other animal models. Sildenafil reduced the elevated serum creatinine concentration in rats following 5/6 nephrectomy<sup>[35]</sup>, while another PDE 5 inhibitor normalized the level in SHR treated with cyclosporin A<sup>[32]</sup>. These findings demonstrate the beneficial effect PDE 5 inhibitors have on impaired renal function.

## HAEMODYNAMIC FACTORS

### Glomerular filtration rate/creatinine clearance

Glomerular filtration rate (GFR) and creatinine clearance (CrCl), a good index of GFR<sup>[56]</sup>, are routinely used to check kidney function. Specifically, they estimate blood flow per minute through the glomeruli, and measure how well the kidneys filter the DN-induced build up of blood creatinine. The early stages of DN are characterized by an increase in glomerular hyperfiltration, which elevates GFR and contributes to renal impairment<sup>[37]</sup>. However, as the disease progresses, renal function deteriorates and there can be a relentless decline in GFR<sup>[16,38,39]</sup>. This functional change develops as a consequence of structural abnormalities, including an increase in kidney size<sup>[29,31]</sup>, together with poor metabolic control<sup>[40]</sup>. Lau *et al*<sup>[34]</sup> found CrCl was reduced in diabetic rabbits and restored by vardenafil, suggesting that PDE 5 inhibitors can improve diabetes-induced renal impairment.

Cyclosporin A-induced nephrotoxicity and renal damage caused by 5/6 nephrectomy, provide indirect support for this concept; both are characterized by a decrease in CrCl and improved by treatment with a PDE5 inhibitor<sup>[32,35]</sup>.

The beneficial effect of the PDE 5 inhibitors is likely to be due to NO-cGMP accumulation<sup>[20,21]</sup> causing dilatation of glomerular afferent blood vessels<sup>[19]</sup>. In this regard, it is proposed that glomerular hyperfiltration depends upon an increase in NO activity in the early phase of DN<sup>[41]</sup>, whereas in the later phase when the GFR starts to fall, a concomitant reduction in NO activity would lead to glomerular hypertension. The diabetes-induced reduction in NO activity could be due to defective synthesis or quenching through the production of superoxide radicals and AGEs<sup>[42,43]</sup>. Therefore, the beneficial action of PDE 5 inhibitors is to increase renal NO-cGMP activity and restore GFR/CrCl. An increase in kidney cGMP content, rather than blood pressure reduction, was also thought responsible for the improved renal function in rats with cyclosporin A-induced nephrotoxicity following PDE 5 inhibitor treatment<sup>[32]</sup>.

Hypertension is twice as frequent in diabetic patients and a major reason why most develop cardiovascular disease<sup>[44]</sup>. It also plays a significant role in the progression of DN<sup>[7,45]</sup>. Consequently, lowering blood pressure has to be an important consideration in the management of DN. Kuno *et al*<sup>[29]</sup> noted that sildenafil treatment for 8 wk significantly reduced systolic and diastolic blood pressure in OLETF rats, providing further evidence of the haemodynamic benefits that can be achieved by treatment with PDE 5 inhibitors.

### Urinary albumin excretion and total protein/creatinine ratio

The progressive increase in urinary albumin excretion, commonly termed proteinuria is another clinical hallmark of DN and also a predictor of cardiovascular disease. Such DN-induced increase in proteinuria is part of a series of clinical events, which includes elevated blood pressure and a progressive decline in GFR. Moreover,

proteinuria does not diminish as DN progresses. Proteinuria is, therefore, a consequence of the glomerular damage in diabetes mellitus and a cause of further damage, since it leads to inflammation and fibrosis in the renal tubules and a loss of functional nephrons. Urinary albumin excretion and total protein/creatinine ratio can be used to monitor proteinuria<sup>[46,47]</sup>. Both markers were elevated in six months diabetic rabbits, as well as diabetic and OLETF rats and were normalized by vardenafil and sildenafil treatment<sup>[28,29,34]</sup>. Sildenafil also reduced the elevated proteinuria in 5/6 nephrectomized rats<sup>[35]</sup>. Taken together, these findings imply that PDE 5 inhibitors reduce proteinuria and improve the renal status in DN.

### Inflammation and fibrosis

Diabetes-induced kidney fibrosis results from prolonged renal injury initiated by excessive extracellular matrix deposition. Inflammation is central to the development and progression of this complication. It is characterized by glomerular and tubulointerstitial migration of activated inflammatory cells (neutrophils, macrophages, T lymphocytes, and mast cells) and fibroblasts in the kidney, regardless of the initial insult. At the injury site, inflammatory cells synthesize reactive oxygen species plus fibrogenic cytokines and growth factors, which exacerbate the renal damage. This leads to excessive and poorly ordered matrix deposition and fibrosis; in turn this affects normal-tissue architecture and ultimately can disable proper functioning of the kidney<sup>[9,48]</sup>. Jeong *et al*<sup>[28]</sup> found that sildenafil treatment significantly attenuated the diabetes-induced increase in renal cortex 8-OHdG content and its elevated excretion (measures of oxidative stress and DNA damage), probably by inhibiting the accumulation of oxidized DNA in the kidney. DN-induced macrophage infiltration into the glomeruli and tubulointerstitium of diabetic rats, an indication of inflammation, was also ameliorated by sildenafil. It seems likely that controlling excessive inflammation and generation of reactive oxygen species with PDE 5 inhibitors will have therapeutic potential in inhibiting diabetes-induced kidney fibrosis. Interestingly, drugs with anti-inflammatory activity have been found to slow or reverse DN<sup>[9]</sup>.

### Angiotensin II

Angiotensin II (Ang II), is an octapeptide trophic hormone and a powerful vasoconstrictor widely recognised as a regulator of blood pressure, fluid and electrolyte homeostasis<sup>[7]</sup>. It has a central role in the pathogenesis of DN where increased Ang II levels cause a preferential constriction of the efferent glomerular arterioles, an increased glomerular permeability to proteins and enhanced formation of AGEs<sup>[33]</sup>. It regulates, therefore, systemic and glomerular haemodynamics, as well as glomerular hypertrophy and sclerosis<sup>[7]</sup>. In mesangial cells it triggers the production and release of cytokines, chemokines and growth factors, with the net effect of mediating and/or amplifying renal damage<sup>[18]</sup>. The physiological actions of Ang II in the kidney are due to activation of angiotensin II receptor type 1

(AT1) receptors. These receptors are mainly expressed in smooth muscle cells where they induce vasoconstriction, proliferation and inflammation<sup>[21]</sup>.

The regulation of Ang II is governed by interplay with NO<sup>[21]</sup>. NO antagonizes the vasoconstrictive and pro-atherosclerotic effect of Ang II, as well as directly modulating angiotensin-converting enzyme (ACE) activity. Conversely, Ang II decreases NO bioavailability by promoting oxidative stress<sup>[21,49]</sup>. Ang II has been reported to upregulate super oxide production in endothelial and vascular smooth muscle cells, which is thought to directly contribute to Ang II-induced contraction of vascular smooth muscle<sup>[50]</sup>.

It is possible that the beneficial effect of PDE 5 inhibitors in DN is due to their regulatory action on the renal Ang II response. There is clinical evidence that inhibition of ACE and AT1 receptor blockade suppress development of DN<sup>[51-53]</sup>. Whether the beneficial effects of PDE 5 inhibitors in DN also include regulatory actions on the renal Ang II response merits future investigation.

## CONCLUSION

Over the last few years the cellular and molecular events underlying the renal structural and functional abnormalities of DN have been the subject of intense study. The role played by metabolic and haemodynamic stimuli in disease progression should not be underestimated. It is likely that diabetic hyperglycaemia and oxidative stress increase the formation of AGEs, cytokines and growth factors, which are important in the development of glomerulosclerosis and tubulointerstitial fibrosis, by stimulating the production of extracellular matrix and inhibiting its degradation. Ang II also plays an important role in DN, since it increases mesangial proliferation and matrix accumulation and induces proinflammatory and fibrogenic processes. These events lead to the progressive decline of nephron function and their destruction.

The evidence outlined in this review suggests that PDE 5 inhibition may provide an additional therapeutic option to treat this debilitating disorder. Ultimately, it may be necessary to use PDE5 inhibitors in conjunction with other treatment modes; for example, hypoglycaemic drugs and antihypertensives, such as ACE inhibitors and AT1 receptor blockers. In addition, cardiovascular risk factors, such as hyperlipidaemia and smoking should be reduced. These life style changes, supported by appropriate drug therapy, could ultimately reduce the number of patients with DN who require costly renal dialysis or transplantation.

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## Type 1 diabetes and celiac disease: The effects of gluten free diet on metabolic control

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### Abstract

Type 1 diabetes mellitus is associated with celiac disease, with a prevalence that varies between 0.6% and 16.4%, according to different studies. After a diagnosis of celiac disease is confirmed by small bowel biopsy, patients are advised to commence a gluten-free diet (GFD). This dietary restriction may be particularly difficult for the child with diabetes, but in Europe (and in Italy) many food stores have targeted this section of the market with better labeling of products and more availability of specific GFD products. Treatment with a GFD in symptomatic patients has been shown to improve the symptoms, signs and complications of celiac disease. However, the effects of a GFD on diabetic control are less well established. Initial reports of improved hypoglycemic control were based on children who were diagnosed with celiac disease associated with malabsorption, but there have subsequently been reports of improvement in patients with type 1 diabetes with sub-clinical celiac disease. There are other studies reporting no effect, improved control and an improvement of hypoglycemic episodes. Moreover, in this review we wish to focus on low glycemic index foods, often suggested

in people with type 1 diabetes, since they might reduce postprandial glycemic excursion and enhance long-term glycemic control. In contrast, GFD may be rich in high glycemic index foods that can increase the risk of obesity, insulin resistance and cardiovascular disease, worsening the metabolic control of the child with diabetes. Hence, it is important to evaluate the impact of a GFD on metabolic control, growth and nutritional status in children with type 1 diabetes.

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**Key words:** Adolescents; Celiac disease; Children; Glycemic control; Type 1 diabetes

**Core tip:** It is important to evaluate the impact of a gluten-free diet (GFD) on metabolic control, growth and nutritional status in children with type 1 diabetes and celiac disease. Since compliance with a strict GFD and a safe choice of food for diabetes is not easy, these patients require extra education and dietary intervention. A specialized follow-up and dietary counseling are essential in the management of patients affected by both type 1 diabetes and celiac disease.

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### INTRODUCTION

Type 1 diabetes is an immune-mediated disorder characterized by a deficit or absence of insulin resulting from T cell-mediated destruction of beta cells of the pancreas<sup>[1]</sup>.

Children with type 1 diabetes have an increased risk of developing other autoimmune disorders like Hashi-

moto's thyroiditis, Addison disease, vitiligo and celiac disease<sup>[2]</sup>. The relation between type 1 diabetes and these pathologies is a common genetic background. All of these diseases are associated with organ-specific autoantibodies that can be detected before the development of clinical diseases; consequently, patients affected by type 1 diabetes usually undergo a scheduled (usually once a year) screening for these pathologies.

Celiac disease is one of the most common autoimmune disease-based disorders; it is elicited by a failure of oral tolerance towards wheat, gluten and related cereals, which results in a multisystem inflammation of the intestinal tract. It usually develops in HLA-DQ2/8 positive individuals. The first association between type 1 diabetes and celiac disease was suggested in 1969<sup>[3]</sup>. The genetic risk factors associated with both diseases include human leukocyte antigen (*HLA*) genes and non-*HLA* genes.

The increased prevalence of celiac disease in patients with type 1 diabetes is due to an overlap in the genetic susceptibility to both diseases conferred by the *HLA-DR3/DQ2*<sup>[4]</sup>. This haplotype is present in over 90% of patients with celiac disease and 55% of those with type 1 diabetes, compared with only 20%-25% of the general population of European ancestry. HLA-DQ8 also confers a risk of type 1 diabetes<sup>[4]</sup>.

Celiac disease affects at least 10% of patients with type 1 diabetes at some point in their lives<sup>[5]</sup>, with a prevalence that varies between 0.6%-16.4%, according to different studies<sup>[6-8]</sup>. The prevalence of celiac disease among children with type 1 diabetes is significantly higher than in non-diabetic children (in Western countries celiac disease affects around 1%-2% of the non-diabetic population).

In Italy, the prevalence of celiac disease in children with type 1 diabetes is around 7%<sup>[9,10]</sup>, 3.6% of which at type 1 diabetes onset<sup>[9]</sup>, at a younger age<sup>[10]</sup> and in boys<sup>[10]</sup>; moreover, according to a study by Salardi *et al*<sup>[11]</sup>, the prevalence of celiac disease has significantly increased since 1994 (10.6% *vs* 6.6%,  $P = 0.015$ ), probably due to changes in environmental factors, namely, eating habits and viral infections.

Less than 10% of patients with type 1 diabetes who develop celiac disease show gastrointestinal symptoms, while most of the children are either asymptomatic or only mildly symptomatic. Therefore, children affected by type 1 diabetes undergo screening for celiac disease. Usually, celiac autoantibodies are tested at the time of diabetes onset and yearly during follow-up, but debate exists about timing and frequency for screening<sup>[12,13]</sup>. When celiac antibodies are detected (ideally confirmed at least twice), it is mandatory to perform esophagogastroduodenoscopy with bowel biopsies to confirm diagnosis<sup>[14]</sup>.

## TREATMENT OF CELIAC DISEASE FOR PATIENTS WITH TYPE 1 DIABETES

The presence of mucosal atrophy is an indication to start a gluten-free diet (GFD), which is the standard therapy for celiac disease, avoiding all foods containing wheat, rye,

barley and oats.

Patients affected by celiac disease must follow a strict GFD for their entire life to prevent acute (malabsorption, diarrhea, folate deficiency, failure to thrive, iron deficiency) and chronic (intestinal lymphoma, osteoporosis, autoimmune diseases, infertility, mortality) complications<sup>[12,15,16]</sup>.

Gluten restriction added to a diabetic dietary regimen imposes practical limitations and leads to considerable restrictions in the lifestyle of a child or adolescent. Unfortunately, as a result, non-adherence to GFD among patients with type 1 diabetes and celiac disease is very common. A study by Valerio *et al*<sup>[17]</sup> found that only 59% of patients with type 1 diabetes and celiac disease were compliant to a strict GFD, while compliance in patients with celiac disease only is around 78%<sup>[18]</sup>. This is an important factor to consider when treating a child or adolescent with type 1 diabetes. It is well established that an accurate diet is one of the cornerstone of the management in patients with type 1 diabetes<sup>[19]</sup>. Combining a GFD may raise major challenges and even some doubts. Dietary intervention aims to achieve and maintain blood glucose and blood pressure in the normal range, to attain normal lipid profile, to achieve normal body weight<sup>[19]</sup>. Preserving a steady glycemic control is essential to reduce both micro and macrovascular complications of type 1 diabetes<sup>[20]</sup>. For this reason, it is important to give patients advice on carbohydrate amount, type and distribution throughout the day, and to educate them about carbohydrate counting. In this context, the choice of low glycemic index food may be important<sup>[21]</sup>. In this respect, a GFD could be an obstacle as many of the gluten-free foods have a high glycemic index. This might influence glycemic values, HbA1c, insulin requirement, lipid profile, and possibly the development of long-term diabetic complications. Moreover, GFD could modify both anthropometric measures, such as height, weight, body mass index (BMI), growth velocity, even if not all researchers agree on the final effects of GFD.

## BODY MASS INDEX IN CHILDREN WITH TYPE 1 DIABETES AND CELIAC DISEASE

While, in patients with celiac disease alone, concern has been raised about gaining weight when on a GFD<sup>[22]</sup>, recent data show normal growth patterns in children and adolescents with type 1 diabetes and celiac disease<sup>[23]</sup>, with body mass index and height standard deviation scores only marginally but not significantly higher in the control (non-celiac) than the study group, and similar to subjects with celiac disease with good or fair/poor adherence to a GFD throughout the follow-up period. Among the reasons for increased BMI, the macronutrient composition of gluten-free foods, a high percentage of saturated fat and carbohydrates with high glycemic index, and a low percentage of proteins and fiber can be included.

After clearing gluten, as villous atrophy resolves, intestinal absorption is certainly improved, but an excessive weight gain may increase the risk of morbidity and may

lead to higher risk of cardiovascular disease<sup>[24]</sup> especially in type 1 diabetes patients. However, data on weight gain (and BMI increasing) in patients with celiac disease are inconsistent. Dickey *et al.*<sup>[22]</sup> showed that nearly 80% of patients gained some weight after 2 years on GFD, and about 51% were even overweight or obese. On the contrary, a recent study reported a weight loss in obese or overweight patients while on GFD<sup>[25]</sup>, with a similar improvement in screen- and symptom-detected celiac disease patients on a GFD.

## GLYCEMIC CONTROL IN CHILDREN WITH TYPE 1 DIABETES AND CELIAC DISEASE

Regarding patients with type 1 diabetes and celiac disease, the most recent data show no difference between patients with and without celiac disease<sup>[23]</sup>. However, a link between a change in body mass index and a possible improvement of metabolic control remains controversial. Acerini *et al.*<sup>[26]</sup> observed an improvement both in body mass index and in HbA1c, while Nóvoa Medina *et al.*<sup>[27]</sup>, who studied only type 1 diabetes patients with symptomatic celiac disease, did not find any effects on metabolic control or on height or weight.

Other studies evaluated the influence of a GFD on metabolic parameters, such as insulin dose, HbA1c, glucose excretion and hypoglycemic episodes. Saadah *et al.*<sup>[28]</sup> observed that a GFD resulted in a significant improvement of growth and influenced diabetic control (more insulin in celiac disease patients when compared to baseline). Other authors<sup>[26,29]</sup> did not find any significant difference in insulin dose, HbA1c, 24 h urinary glucose excretion, or number of hypoglycemic episodes. Similar findings have been observed in adult patients with type 1 diabetes and celiac disease<sup>[30]</sup>. Abid *et al.*<sup>[31]</sup> documented in type 1 diabetes children with celiac disease that a GFD showed short-term benefits by reducing gastrointestinal symptoms and, in particular, episodes of severe hypoglycemia, while there was no change in standard deviation score for height, weight, and BMI or the mean HbA1c before and after GFD. The mean insulin requirement significantly increased. More refined indexes of an altered or better metabolic control, like continuous glucose monitoring, glycemic variability indexes, and frequency of insulin dose changes are usually difficult to measure.

## TYPE 1 DIABETES, CELIAC DISEASE AND MICRO OR MACROANGIOPATHIC COMPLICATIONS

Few studies have been published about this topic and almost all involved adult patients with type 1 and celiac disease. Bakker *et al.*<sup>[32]</sup> collected HbA1c before celiac disease diagnosis, at diagnosis and the most recent together with the presence of nephropathy and retinopathy. An interesting finding was that diabetes patients with celiac disease had a lower prevalence of retinopathy when

compared to controls (diabetes patients without celiac disease), whereas no difference in the prevalence of nephropathy was found, suggesting that a GFD possibly favorably affects the development of vascular complications in diabetes patients.

Similar findings have also been observed about macrovascular complications. Picarelli *et al.*<sup>[33]</sup>, evaluated whether the presence of celiac disease in a group of type 1 diabetes patients is associated with different expression of some hemostatic factors and with a different manifestation/progression of complications. The authors claim a potential protective role of celiac disease in the prothrombotic state of type 1 diabetes (celiac disease patients had significantly lower HbA1c, total cholesterol, triglycerides, factor VII antigen, factor VII coagulant activity, and prothrombin degradation fragments). In contrast, Pitocco *et al.*<sup>[34]</sup> found that in type 1 diabetes patients with long duration of celiac disease, the carotid intima-media layer was thicker compared to diabetes patients without celiac disease.

However, if GFD seems to have a protective role in the appearance of micro- and macroangiopathic complications, the misdiagnosis of celiac disease in adult patients with type 1 diabetes is associated with a higher prevalence of retinopathy, nephropathy and peripheral neuropathy<sup>[35]</sup>. These findings raise the issue of regular celiac disease screening in order to detect type 1 diabetes patients at risk of developing celiac disease in a timely manner.

In this context, the case reported by Sildorf *et al.*<sup>[36]</sup> of a 6-year-old boy who, after type 1 diagnosis, even without celiac disease, was started on a GFD, gradually suspending insulin therapy and remaining free of exogenous insulin after 20 mo seems very interesting. The GFD was reported to be safe and without side effects, and it is believed that the GFD acted to prolong the remission phase of diabetes.

## TYPE 1 DIABETES, CELIAC DISEASE AND GLYCEMIC INDEX

As stated above, the most difficult factor to handle for a child/adolescent with type 1 diabetes and celiac disease is that most GFD foods have a high glycemic index. Indeed, in 2002 the American Society for Clinical Nutrition compared many foods regarding their glycemic index. What they discovered was that gluten-free foods have a higher glycemic index than gluten-containing equivalents. Since glycemic index represents a direct measure of carbohydrate absorption, it is obvious that high glycemic index foods determine a rise in rapid blood glucose values. Hyperglycemia causes an increase in free fatty acids that induce oxidative stress and promote atherosclerosis<sup>[37]</sup>. On the other hand, the subsequent rapid fall in glucose removal is associated with a sensation of hunger and excessive caloric intake<sup>[38]</sup>. Thus, a diet with low glycemic index is suggested either because of a lack of normal insulin response to high glycemic index foods in diabetes patients, or because of the aim of reducing micro and macrovascular complications<sup>[21,39]</sup>. Indeed, we have seen that GFD seems to have a protective role rather than a

deteriorating one<sup>[32,33]</sup>, even in pediatric age<sup>[40]</sup>. The means by which the presence of celiac disease might prevent micro- and macrovascular complications of diabetes asks further investigations. Hypothetically, a greater dietary vigilance, an increased awareness of food intake and several consultations by a skilled dietitian might result in a better controlled carbohydrate intake and could lead to healthier eating habits. Finally, gluten free foods have a reduced content of many micronutrients: B and D vitamins, calcium, iron, magnesium and zinc. In particular, calcium content in a GFD should be appropriate, since an impairment of bone metabolism and structure has been found both in type 1 diabetes and celiac disease.

## CONCLUSION

Hence, it is important to evaluate the impact of a GFD on metabolic control, growth and nutritional status in children with type 1 diabetes and celiac disease.

Since compliance with a strict GFD and a safe choice of food for diabetes is not easy, these patients require extra education and dietary intervention.

A specialized follow-up and dietary counseling are essential in the management of patients affected by both type 1 diabetes and celiac disease.

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## Association of comorbidities with increasing severity of peripheral neuropathy in diabetes mellitus

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### Abstract

**AIM:** To analyze a large population of patients with diabetes and peripheral neuropathy (PN) to determine other meaningful comorbid etiologies for PN.

**METHODS:** Peripheral Neuropathy is a common complication of type 1 and 2 diabetes mellitus; however, other potential causes for PN may be co-existing in patients with diabetes. A prospective cohort study was performed to assess patients with diabetes and PN. We compared patients having PN due solely to diabetes with patients possessing co-existing comorbidities, performing clinical (Toronto Clinical Scoring System and the Utah Early Neuropathy Scale), laboratory and electrophysiological assessments in all patients.

**RESULTS:** Patients with either type 1 or 2 diabetes mellitus and co-existing comorbidities did not have more severe clinical or electrophysiological PN phenotypes overall. However, in patients with type 1 diabetes, presence of a lipid disorder was associated with greater PN severity. In type 2 diabetes patients, both a lipid

disorder and cobalamin deficiency were associated with greater PN severity. There was no additive effect upon PN severity with presence of three or more comorbid etiologies.

**CONCLUSION:** The presence of specific, and not general, comorbidities in patients with type 1 or 2 diabetes corresponds with greater PN severity.

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**Key words:** Diabetic peripheral neuropathy; Comorbidities; Lipidemia; Cobalamin; Methylmalonic acid

**Core tip:** The cause of diabetic peripheral neuropathy (DPN) has remained elusive. Comorbid conditions may contribute to the severity of DPN. We studied patients with type 1 or 2 diabetes and concurrent DPN in order to identify potential comorbid conditions associated with greater neuropathic deficit. Concurrent lipidemia was associated with worse DPN in either type 1 or 2 diabetes. A concurrent vitamin B12 deficiency increased severity of DPN in type 2 diabetes. Although our results were potentially confounded by higher HbA1C values in patients with comorbid conditions, vigilance should occur for other causes of PN when diabetes is present.

Sachedina S, Toth C. Association of comorbidities with increasing severity of peripheral neuropathy in diabetes mellitus. *World J Diabetes* 2013; 4(4): 135-144 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i4/135.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i4.135>

### INTRODUCTION

Peripheral neuropathy (PN) is a prevalent condition in the general population<sup>[1]</sup>. While the most common cause of PN is diabetes mellitus, of both type 1 and type 2

forms, there are many other proven etiologies and forms of PN. Patients with diabetes are subject to comorbid conditions, either by association or coincidence. As such, patients with PN due to diabetes [termed diabetic peripheral neuropathy (DPN)] may manifest other conditions capable of exacerbating or initiating PN. Although different etiologies of PN possess various pathophysiologies, the presence of PN and its increasing severity greatly reduces quality of life<sup>[2]</sup>. Clinically, patients presenting with symptoms of PN in the presence of already or newly diagnosed diabetes are often subsequently concluded to have only DPN without further laboratory investigations performed. This may preclude investigations to determine other potential, and sometimes treatable, causes of PN. The aim of this study was to identify those patients with the presence of multiple conditions capable of causing PN other than diabetes to determine if multiple comorbidities increases PN severity. We hypothesized that the presence of comorbidity capable of leading to PN occurring in conjunction with either type 1 or 2 diabetes would lead to an increase in severity of PN. Further, we hypothesized that the presence of multiple comorbidities would have an additive effect upon the severity of PN.

Particular comorbidities have shown relationship to greater severity of DPN, and have included elevated triglycerides, smoking, hypertension, and obesity<sup>[3]</sup>. Hyperlipidemia<sup>[4]</sup> and statin medication use<sup>[5,6]</sup> are both exceedingly common in patients with diabetes, and may also be implicated as causative for PN. Another recent association is that of metformin use, which was associated with elevation of fasting methylmalonic acid levels and greater presence of DPN<sup>[7]</sup>; this association may relate to a resulting vitamin B12 (cobalamin) deficiency. At present, we are not aware of other potential comorbidities important in the assessment of DPN patients. Therefore, in the current study, we sought for any additional comorbidities capable of contributing to the greater impact and severity of DPN.

We designed this prospective study to examine our hypotheses and to detect any clinically meaningful synergistic effects of comorbid conditions in patient populations with diabetes mellitus. We assessed for presence of both general and specific comorbidities, including alcoholism, thyroid disease, monoclonal gammopathy of uncertain significance, autoimmune antibody presence, uremia, and cobalamin or other vitamin deficiencies with or without associated high fasting methylmalonic acid levels. We concurrently examined hypercholesterolemia and hyperlipidemia (grouped as a lipid disorder), and hypertension, all of which are potential risk factors for the development of DPN<sup>[3]</sup>.

## MATERIALS AND METHODS

### Subject recruitment

This study was ethically approved by the University of Calgary Centre for Advancement of Health. Recruitment of subjects occurred from December 2008 until July 2010

at the Neuromuscular and Neuropathic Pain Clinics at the University of Calgary. Subjects were recruited prospectively upon initial evaluation at the tertiary care clinics. Inclusion criteria consisted of the following: (1) all subjects provided informed written consent prior to involvement; and (2) a diagnosis of pre-existing type 1 or 2 diabetes was provided based upon laboratory testing—two prior fasting glucose results of  $\geq 7.1$  mmol/L (126 mg/dL) [or random glucose of  $\geq 11.1$  mmol/L (200 mg/dL) with symptoms of hyperglycemia for type 1 diabetes] or two oral glucose tolerance tests leading to a 2 h serum glucose of  $\geq 11.1$  mmol/L (200 mg/dL) (based on Canadian Diabetes Association guidelines). Exclusion criteria included: (1) subjects with impaired fasting glucose or impaired glucose tolerance; and (2) absence of discernible PN or presence of questionable PN (see below). The age of diagnosis of diabetes and the duration of symptoms of PN were recorded. There was no specific sample size calculation performed and no pre-specified cohort patient number was determined for this study.

### Clinical assessment of peripheral neuropathy

Clinically, each patient was examined for PN and scored using the Toronto Clinical Scoring System (TCSS)<sup>[8]</sup> and the Utah Early Neuropathy Scale (UENS)<sup>[9]</sup>. The TCSS is a validated method for evaluation of PN with higher scores correlated with greater sural nerve pathology on biopsy<sup>[8]</sup>. The TCSS has greater emphasis upon sensory deficits related to PN as compared with other comparable scales. All clinical examination was performed prior to knowledge of blood testing results. The UENS has greater applicability to determining clinical progression of PN than TCSS, and also places emphasis upon sensory abnormalities. After clinical scales were completed, subjects with TCSS  $\leq 5$  and UENS  $\leq 6$  were excluded due to uncertainty regarding the presence of PN. Subjects receiving known neurotoxic medications or chemotherapy, or with a history of carcinoma were excluded. Although laboratory testing was performed after clinical evaluation, the evaluator was not blinded to the form of diabetes mellitus or the presence of previously diagnosed comorbid conditions.

### Laboratory assessment of peripheral neuropathy

Laboratory testing (Calgary Laboratory Services) was performed after clinical evaluation, and consisted tests listed in Table 1. When abnormalities were identified with blood testing, those particular abnormal tests were repeated using new blood samples for verification. Past or present alcoholism was also taken into account and diagnosed based upon Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria. Seated blood pressure measurements were performed twice—hypertension was based upon two measurements  $\geq 130/80$  using the Canadian Diabetes Association criteria or based upon pre-existing diagnosis made prior to evaluation.

Diagnosis of comorbidity was provided based upon an identified laboratory abnormality or previously identified

**Table 1 Blood testing performed for identification of comorbidities**

Blood test	Normative range
Red blood cell counts	
Women	4.0-5.6 × 10 <sup>12</sup> /L
Men	4.5-6.0 × 10 <sup>12</sup> /L
White blood cell counts	4.0-11.0 × 10 <sup>9</sup> /L
Platelet counts	150-400 × 10 <sup>9</sup> /L
Electrolytes (mmol/L)	
Sodium	133-145
Potassium	3.3-5.1
Chloride	98-111
Bicarbonate	21-31
Calcium	2.10-2.55
Magnesium	0.65-1.05
Urea (mmol/L)	3.0-8.5
Creatinine (μmol/L)	50-120
Aspartate transaminase (U/L)	8-40
Alanine transaminase (U/L)	1-60
Gamma glutamyl-transferase (U/L)	8-40
Albumin (g/L)	33-48
Total bilirubin (μmol/L)	0-20
Total cholesterol (mmol/L)	3.8-5.2
Low density lipoproteins (mmol/L)	2.2-3.4
High density lipoproteins (mmol/L)	> 0.9
Triglycerides (mmol/L)	0.6-2.3
Cobalamin (measured by immunoassay), (pmol/L)	> 155
Thiamine (μg/L)	33-110
Thyroid stimulating hormone (mU/L)	0.2-6.0
Total thyroxine (nmol/L)	59-154
Antinuclear autoantibody detection	≤ 1:80
Extractable nuclear antigens	Absent
Serum protein electrophoresis	The sensitivity for detection of gammopathy was 2 g/L with serum protein electrophoresis, and samples with peaks of 2-4 g/L were subjected to immunofixation for verification
Serum copper (μmol/L)	11-24
Fasting methylmalonic acid (measured using high performance liquid chromatography), (μmol/L)	< 0.15
Hemoglobin A1C	4.3%-6.1%

Normative data was supplied by the laboratory that performed all related testing-Calgary Laboratory Services in Calgary, Alberta, Canada. Normative data is presented as a range based upon 95%CI.

comorbidity diagnosed prior to this evaluation. Previously diagnosed comorbidities were verified using electronic or paper patient chart information. All abnormalities were determined using the population normal values as determined by Calgary Laboratory Services.

Once the presence of underlying comorbidities was determined, subjects were categorized into four groups: patients solely with type 1 diabetes (DM1 only); patients with type 1 diabetes and an existing comorbidity or comorbidities (DM1 plus comorbidity); patients solely with type 2 diabetes (DM2 only); and patients with type 2 diabetes and an existing comorbidity or comorbidities (DM2 plus comorbidity). Sub-categorization based upon individual comorbidities and number of comorbidities was performed subsequently. The following comorbidi-

ties were used for categorization: lipid disorder (elevated low density lipoprotein or triglycerides or both), cobalamin deficiency (depressed cobalamin level or elevated fasting MMA level or both), monoclonal gammopathy of uncertain significance (MGUS), thyroid disorder, renal dysfunction (elevated creatinine), autoimmune disorder [prior diagnosis or detection of positive extractable nuclear antigen (ENA) status of significance], alcoholism, or hypertension. We defined an autoimmune disorder as the presence of an inappropriate immune response with detectable auto-antibodies having potential for leading to a neurological disease including rheumatoid arthritis, sjogren syndrome, systemic lupus erythematosus, or systemic vasculitis. Other potential comorbidities were recorded for consideration of potential impact.

### **Electrophysiological assessment of peripheral neuropathy**

All patients received electrophysiological evaluation for PN severity after clinical evaluation and prior to receipt of laboratory testing results. Cadwell Sierra Wave (Cadwell Laboratories, Kennewick, WA) electromyography machines were used. Both motor and sensory testing was performed on the non-dominant upper and lower limb; in the case of ambidextrous patients, the left upper and lower limbs were studied. Motor nerve conduction studies (NCS) were performed using stimulation of the median nerve (wrist, elbow), ulnar nerve (wrist, below elbow, above elbow), peroneal (ankle, below fibular head and above fibular head locations) and tibial (ankle, popliteal fossa locations) nerves. For each motor nerve, distal motor latencies, compound motor action potentials, and conduction velocities were obtained or calculated. F wave latencies were obtained from median, ulnar, peroneal and tibial nerves. Sensory antidromic NCS were performed using the median (digits 2 and 4), ulnar (digits 4 and 5), superficial radial, superficial peroneal and sural nerves, with sensory nerve action potentials (SNAP), onset latency, and conduction velocity obtained or calculated. Temperatures were maintained at ≥ 32 °C for the upper extremities, and ≥ 30 °C for the lower extremities during NCS testing. Absent electrophysiological responses were used to calculate amplitude values, but latency and velocity values were not entered in the analysis for absent responses in order to not obscure data analysis. Patients were excluded if they refused electrophysiological testing or laboratory testing.

### **Statistical analysis**

Our primary objective was to determine the impact of presence of any comorbidities associated with development of PN upon the severity of PN in patients with either type 1 or 2 diabetes. Secondly, we also analyzed specific individual comorbidities, and presence of multiple comorbidities for determination of impact upon PN severity. Analysis was performed for the subject categorizations described above. Group equivalence for age, duration of diabetes, duration of PN symptoms, A1C,

and alcohol exposure were compared by independent samples *t*-test; gender was compared by chi-square testing. In all cases, type 1 and 2 diabetes were considered separately and the two forms of diabetes were not directly compared. Other elements of the past medical history not specified above were not statistically compared due to their heterogeneity. Our primary outcome measures were clinical neuropathy severity (TCSS, UENS) and electrophysiological markers of neuropathy; for the latter we chose to test sensory NCS of the lower extremity (conduction velocity and SNAP for superficial peroneal and sural nerves) as we hypothesized these would most likely to demonstrate exacerbation due to progression of PN. Secondary outcome variables included the other sensorimotor electrophysiological parameters for motor responses of the lower limbs and sensorimotor studies of the upper limbs. We determined that these data did not follow a normal distribution (performed with Shapiro-Wilk testing) so comparisons were made using Mann-Whitney *U* test. Bivariate correlations of primary outcomes and numbers of comorbidities were calculated using Spearman rho test. In addition, we performed a post-hoc linear regression analysis for determination of any potential associations with worsening diabetic status (using HbA1C). We used HbA1C scores as the dependent variable, while explaining variables were chosen to be fasting Methylmalonic acid (MMA) levels, triglycerides, total cholesterol levels, low density cholesterol, and high density cholesterol. Furthermore, a post-hoc linear regression analysis was performed for the type 2 diabetes patient cohort to determine any potential association between cobalamin and fasting MMA levels with greater severity of PN—for this, we used TCSS and UENS scores as the dependent variables, while explaining variables were chosen to be fasting MMA levels. Lastly, a linear regression analysis was performed using TCSS and UENS total scores as the dependent variable and age, duration of diabetes, A1C and presence of comorbidities and number of comorbidities as explaining variables. We set  $\alpha$  to be 0.05, and we utilized Bonferroni corrections for analysis of secondary outcome measures, applied whenever multiple comparisons for the same cohorts were performed. Values are presented as mean  $\pm$  SE throughout.

## RESULTS

### Subject demographics

Demographics and individual comorbidities for each cohort are presented in Table 2. We prospectively enrolled a total of 369 patients. A total of 32 patients (3 type 1 diabetes, 29 type 2 diabetes) declined participation based upon personal choice. DM1 only and DM1 plus comorbidity cohorts were similar with respect to age, gender, duration of diabetes, and HbA1C. However, DM2 plus comorbidity cohorts had longer durations of diabetes and higher HbA1C levels as compared to the DM2 only cohort. We excluded a total of 10 patients for unwill-

ingness to perform testing. Another 17 patients were excluded due to presence of impaired fasting glucose or impaired glucose tolerance rather than strict diabetes.

### Type 1 diabetes and comorbidities

The presence of an identified comorbidity (Table 2) in patients with type 1 diabetes did not increase the TCSS ( $P = \text{NS}$ ,  $F = 3.1$ ) or UENS ( $P = \text{NS}$ ,  $F = 1.4$ ) scores (Figure 1). In addition, primary electrophysiological outcomes for sensory electrophysiological testing of the lower limbs were also not different between DM1 only and DM1 plus comorbidity cohorts ( $P = \text{NS}$ ,  $F = 0.00-1.2$ ).

For secondary outcome measures, after Bonferroni corrections were applied. Analysis showed DM1 plus comorbidity subjects had increased onset latency for the sensory conduction study at the ulnar nerve at digits 4 and 5 ( $3.3 \pm 0.1$  ms *vs*  $3.6 \pm 0.1$  ms,  $P < 0.001$ ,  $F = 8.9$  and  $3.2 \pm 0.1$  ms *vs*  $3.6 \pm 0.1$  ms,  $P < 0.001$ ,  $F = 10.6$  respectively).

For individual comorbidities, type 1 diabetes patients<sup>[6,10]</sup> with presence of triglyceridemia or lipid disorder had greater TCSS (ANOVA,  $P < 0.007$ ,  $F = 8.4$ ) and UENS (ANOVA,  $P < 0.007$ ,  $F = 13.7$ ) scores (Figure 1) than type 1 diabetes patients without comorbidities. Other individual comorbidities did not impact upon severity of PN. Finally, the presence of multiple ( $\geq 3$ ) comorbidities in type 1 diabetes patients did not have a compounding effect for the severity of PN ( $P = \text{NS}$ ,  $F = 1.2$ ).

### Type 2 diabetes and comorbidities

The presence of an acknowledged comorbidity in patients with Type 2 diabetes did not increase the TCSS ( $P = \text{NS}$ ,  $F = 2.3$ ) or UENS ( $P = \text{NS}$ ,  $F = 2.2$ ) scores (Figure 2). Similarly, there were no electrophysiological differences between DM2 only and DM2 plus comorbidity for sural and superficial peroneal parameters ( $P = \text{NS}$ ,  $F = 0.0-0.9$ ).

For secondary outcome measures, there were significant differences for other electrophysiological parameters. DM2 plus comorbidity subjects did have prolonged onset latency for the median motor studies ( $5.4 \pm 0.1$  *vs*  $4.2 \pm 0.1$ ,  $P < 0.001$ ,  $F = 4.6$ ), peroneal motor studies ( $5.5 \pm 0.1$  *vs*  $5.0 \pm 0.1$ ,  $P < 0.001$ ,  $F = 7.6$ ), and tibial motor studies ( $5.5 \pm 0.2$  *vs*  $4.9 \pm 0.1$ ,  $P < 0.001$ ,  $F = 6.2$ ) when compared to the DM2 only cohort. In addition, there was greater slowing of conduction velocities for the median nerve across the forearm ( $44.3 \pm 0.7$  *vs*  $50.0 \pm 0.5$ ,  $P < 0.001$ ,  $F = 11.5$ ), for the ulnar nerve across the forearm ( $49.3 \pm 1.3$  *vs*  $54.2 \pm 0.8$ ,  $P < 0.001$ ,  $F = 7.7$ ), and for the peroneal nerve across the lower leg ( $35.3 \pm 1.1$  *vs*  $39.3 \pm 0.6$ ,  $P < 0.001$ ,  $F = 5.5$ ) as compared to the DM2 only cohort.

Examination of individual comorbidities again identified the presence of a lipid disorder to contribute to greater PN severity based upon TCSS (ANOVA,  $P < 0.007$ ,  $F = 5.7$ ) and UENS (ANOVA,  $P < 0.007$ ,  $F = 2.5$ ) scores (Figure 2) in type 2 diabetes subjects. In addition, the presence of cobalamin deficiency or elevated fasting MMA levels were associated with higher TCSS (ANOVA,  $P < 0.007$ ,

**Table 2** Demographics for cohorts with diabetes mellitus *n* (%)

	Type 1 diabetes only	Type 1 diabetes plus comorbidity	Type 2 diabetes only	Type 2 diabetes plus comorbidity
Patients	31	19	228	91
Duration of disease (mo)	316 ± 26	310 ± 25	107 ± 7	150 ± 12 <sup>a</sup>
HbA1c level (%)	11.2 ± 1.4	11.7 ± 1.6	9.8 ± 0.2	11.2 ± 0.3 <sup>a</sup>
Age (yr)	50 ± 7	54 ± 8	62.0 ± 1.8	61.0 ± 1.4
Sex (male)	17 (55)	12 (63)	91 (40)	36 (40)
Nature of comorbidities		15 (79)		63 (69)
lipid disorder				
Low cobalamin/elevated MMA		9 (47)		36 (40)
Monoclonal gammopathy		2 (11)		4 (4)
Thyroid disease		5 (26)		11 (12)
Uremia		7 (37)		13 (14)
Autoimmune diseases		4 (21)		8 (9)
Alcoholism		3 (16)		10 (11)
Hypertension		13 (68)		74 (81)
Medications				
Insulin	31 (100)	19 (100)	55 (23)	24 (26)
Metformin			198 (86)	80 (88)
Glyburide			155 (68)	63 (69)
Gliclazide			41 (18)	11 (12)
Statins/Ezetemide	21 (68)	9 (47)	146 (64)	48 (53)
Blood pressure medications	22 (71)	14 (74)	118 (52)	57 (62)
Thyroid replacement	12 (39)	5 (26)	38 (17)	14 (15)
SSRIs	4 (13)	2 (11)	28 (12)	16 (18)
Anxiolytics/Sedatives	8 (26)	3 (16)	48 (21)	13 (14)
NSAIDs	12 (39)	9 (47)	104 (46)	55 (60)
Acetaminophen	6 (19)	3 (16)	37 (16)	17 (19)
Gabapentin	5 (16)	4 (21)	41 (18)	15 (16)
Pregabalin	4 (13)	3 (16)	36 (16)	11 (12)
Codeine	3 (10)	4 (21)	31 (13)	8 (9)
Amitriptyline	3 (10)	3 (16)	25 (11)	6 (7)
Oxycocet	1 (3)	2 (11)	18 (8)	4 (4)
Nortriptyline	1 (3)	1 (5)	12 (5)	4 (4)
Duloxetine	2 (6)	3 (16)	22 (10)	5 (5)
Venlafaxine	3 (10)	2 (11)	25 (11)	4 (4)
Fentanyl	1 (3)	2 (11)	6 (3)	3 (3)
Tramadol	1 (3)	0 (0)	8 (3)	2 (2)
Morphine	0 (0)	2 (11)	7 (3)	3 (3)

<sup>a</sup>*P* < 0.05 *vs* type 2 diabetes only and type 2 diabetes plus comorbidity cohort. MMA: Methylmalonic acid; SSRIs: Selective serotonin reuptake inhibitors; NSAIDs: Nonsteroidal antiinflammatory drugs.

*F* = 3.9) and UENS (ANOVA, *P* < 0.007, *F* = 3.3) scores in type 2 diabetes subjects as compared to DM2 only subjects. Finally, the presence of alcoholism was associated with a higher TCSS (ANOVA, *P* < 0.007, *F* = 2.1) but not UENS (ANOVA, *P* = NS, *F* = 1.6) score in type 2 diabetes cohorts relative to DM2 only subjects.

As with type 1 diabetes, the presence of multiple comorbidities in type 2 diabetes patients did not have an additive effect upon severity of PN.

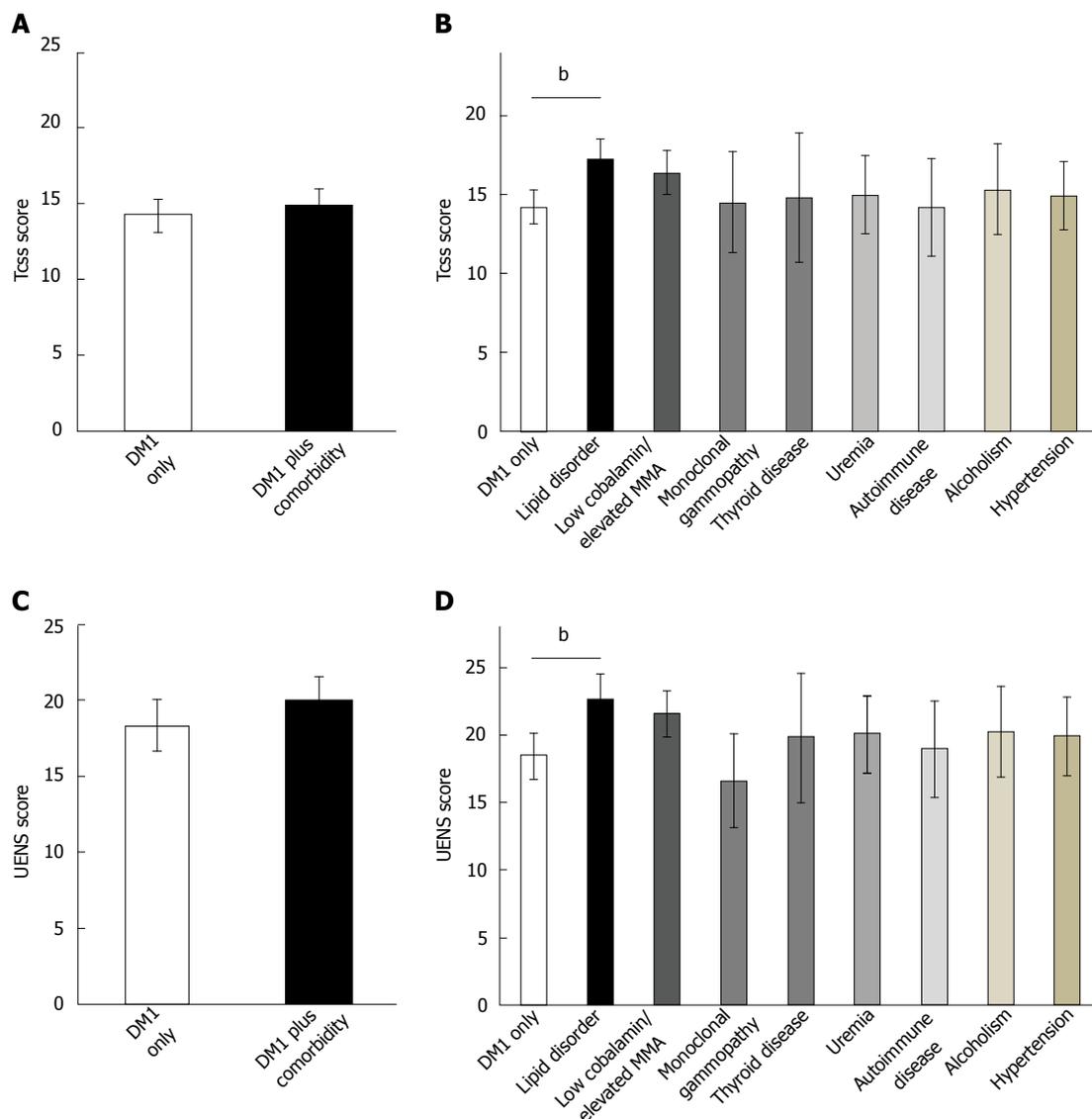
### Multiple comorbidities

We used linear regression to determine the impact of multiple comorbidities upon severity of PN. In type 1 diabetes, there was no significant linear relationship between multiple comorbidities and severity of PN using TCSS (*R*<sup>2</sup> = 0.21) or UENS (*R*<sup>2</sup> = 0.33) scores. Likewise, no additive effect upon PN severity could be shown for type 2 diabetes subjects for multiple comorbidities using TCSS (*R*<sup>2</sup> = 0.04) or UENS (*R*<sup>2</sup> = 0.02) scores.

### Potential associations

We examined for association of potentially important comorbid factors with severity of diabetes mellitus using HbA1C levels. There were no significant associations between HbA1C for either of type 1 or 2 diabetes with any of fasting MMA, triglycerides, total cholesterol, low density cholesterol, or high density cholesterol levels (*R*<sup>2</sup> = 0.08-0.26). However, it should be noted that statin medication use was very common in both type 1 and type 2 diabetic cohorts.

In type 2 diabetes patients, there was a significant association between TCSS and UENS scores with fasting MMA levels (*R*<sup>2</sup> = 0.48 and *R*<sup>2</sup> = 0.52 respectively, *P* < 0.025), and a less robust, but still significant association with cobalamin levels (*R*<sup>2</sup> = 0.42 and *R*<sup>2</sup> = 0.44 respectively, *P* < 0.025). Greater elevation of MMA levels and greater depression of cobalamin levels were associated with greater severity of PN.

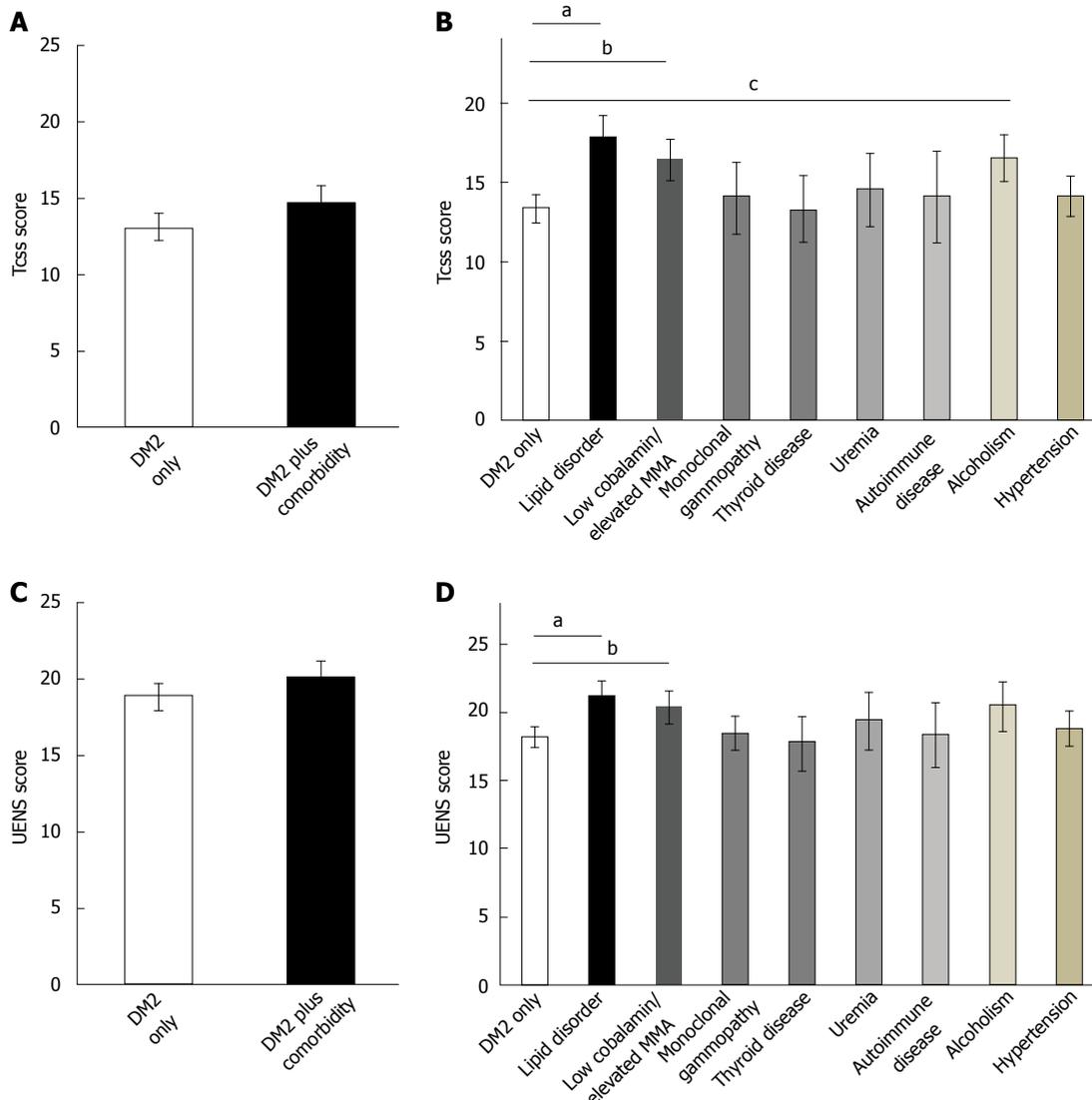


**Figure 1** In subjects with type 1 diabetes. A, B: The level of peripheral neuropathy (PN) severity was measured using the Toronto Clinical Scoring System (TCSS); C, D: The Utah Early Neuropathy Scale (UENS) for patients without (DM1 only) and with (DM1 Plus Comorbidity) comorbidities important for the assessment of PN. There was no significant difference between cohorts for either TCSS (A) or UENS (C) values. However, when examined by specific comorbidity, patients with type 1 diabetes having a concomitant lipid disorder had greater TCSS (B) and UENS (D) values. Other specific comorbidities were not associated with greater severity of PN when compared to DM1 only subjects. The presence of a significant difference between the subcohort with lipid disorder and DM1 only cohort is indicated with (<sup>b</sup>*P* < 0.007 vs lipid disorder, ANOVA).

## DISCUSSION

Although the presence of a coexisting comorbidity did not increase the severity of PN overall in patient cohorts with type 1 or 2 diabetes, particular comorbidities were associated with a more severe phenotype of PN. The presence of a lipid disorder in either type 1 or 2 diabetes was associated with greater neuropathy severity. The presence of cobalamin deficiency and/or elevated fasting MMA levels was correlated with greater presence of neuropathy in type 2 diabetes subjects. While these associations are not necessarily causative, they suggest that greater attention should be afforded to potentially correctable comorbid lipid disorders and for cobalamin deficiencies and/or elevated fasting MMA levels in patients with diabetes. In

our patient populations, we have initiated management of cobalamin deficiencies and elevated fasting MMA levels with continuous monthly intramuscular cobalamin therapy; patients with lipid disorders have simultaneously started on appropriate management. Follow-up data for these interventions is not yet available. The worsening of PN was detected by clinical scoring and not electrophysiological measures for peripheral nerves in the lower extremities, suggesting that worsening of PN may relate to additional small fibre dysfunction, or dorsal column dysfunction in case of cobalamin deficiency, that is undetectable with nerve conduction studies. Another interesting and unexpected finding was the presence of electrophysiological prolongation of latencies and slowing of conduction velocities in DM2 plus comorbidity subjects.



**Figure 2** In type 2 diabetes subjects. A, B: Peripheral neuropathy (PN) severity was assessed using the Toronto Clinical Scoring System (TCSS); C, D: The Utah Early Neuropathy Scale (UENS) for patients without (DM2 Only) and with (DM2 Plus Comorbidity) comorbidities capable of contributing to PN. As with type 1 diabetes, there was no significant difference between cohorts for either TCSS (A) or UENS (C) values. When subcategorized by specific comorbidity, however, patients with type 2 diabetes having a concomitant lipid disorder, cobalamin deficiency or elevated fasting methylmalonic acid level, or alcoholism had greater TCSS (B) scores when compared to DM2 only subjects. Using UENS values (D), presence of a lipid disorder or cobalamin deficiency/elevated fasting methylmalonic acid level was associated with greater PN severity. Other examined comorbidities were not associated with greater severity of PN when compared to DM2 only subjects. The presence of significant differences between the subcohorts with comorbidities and DM2 only cohort are indicated with (<sup>a</sup> $P < 0.007$  vs lipid disorder, ANOVA), (<sup>b</sup> $P < 0.007$  vs low cobalamin or elevated fasting methylmalonic acid level, ANOVA), and (<sup>c</sup> $P < 0.007$  vs alcoholism, ANOVA).

Progression and severity of DPN has been reported to depend upon a number of factors including elevated triglycerides, smoking, hypertension, and obesity<sup>[3]</sup>. Hyperlipidemia may contribute to oxidative stress at the dorsal root ganglia, contributing to greater diabetes-induced neurodegeneration<sup>[11]</sup>. This may also relate to the presence of oxidized low density lipoprotein and its receptor, the lectin-like oxLDL (LOX-1) receptor<sup>[12]</sup>. Possibly toxic to dorsal root ganglia on its own, oxLDL presence, not quantified in this study, is known to be elevated in patients with diabetes and may contribute to other diabetic complications, including retinopathy<sup>[13]</sup>. Previously, there has been speculation about hyperlipidemia leading to peripheral neuropathy irrespective to diabetic status<sup>[4]</sup>, but its occurrence in idiopathic peripheral neuropathy does

not appear to be different from that of control subjects<sup>[14]</sup>. However, hypertriglyceridemia is more common in patients with PN due to diabetes, impaired glucose tolerance, or alcoholism<sup>[14]</sup> so its co-existence is not unexpected. As effective treatments do exist for the management of lipid disorders, future research should assess the potential for intervention in patient populations with DPN. The role of co-existing treatments of lipid modulating drugs, such as statins<sup>[6]</sup>, in the presence of concurrent diabetes requires further investigation as well. Although controversially implicated in peripheral neuropathy<sup>[5,6]</sup>, the role of statins is unclear but they may have played a confounding role in the present study. Lastly, it is possible that patients with concurrent lipid disorders have less rigorous care of their diabetes-patients with DM2 plus comorbidities had a

greater duration of diabetes as well as a higher HbA1C value.

Recently, the presence of elevated MMA levels in patients with DPN has been related to metformin use contributing to greater presence of DPN<sup>[7]</sup>. Moreover, while higher MMA levels are generally related to vitamin B12 (cobalamin) deficiency, this may result from renal dysfunction or elderly age as well<sup>[15]</sup>. Although cobalamin deficiency is most classically associated with subacute combined degeneration, an exclusive peripheral neuropathy (PN) presentation occurs, typically manifesting as an axonal polyneuropathy with additional small fiber dysfunction<sup>[16-18]</sup>. As accumulating evidence suggests that the cobalamin-deficiency-associated metabolite MMA is more sensitive and specific than serum cobalamin itself<sup>[19]</sup>, its use for detection of potential cobalamin deficiency has been recommended as an investigation with high diagnostic yield in patients with distal symmetric polyneuropathy<sup>[20]</sup>. This concurrent deficiency was found in patients with type 2 diabetes, many of whom were taking metformin, with more severe neuropathy phenotypes. In this work, higher levels of fasting MMA or lower levels of cobalamin were also associated with greater severity of PN, as we showed previously in a separate cohort of type 2 diabetes patients<sup>[7]</sup>. However, our findings support the additional and often overlooked assessment for the concurrent presence of cobalamin deficiency, a potentially treatable contribution, in patients with DPN and type 2 diabetes.

Alcoholic polyneuropathy is another form of PN which can be associated with concomitant neuropathic pain. This may be due to the direct toxic effects of ethanol or its metabolites upon peripheral nerve fibers<sup>[21]</sup> or may be related to a subsequent thiamine deficiency<sup>[22]</sup>. Its potential for worsening existing DPN was possibly a factor in the type 2 diabetes population (using the TCSS but not the UENS scale). The presence of alcoholism in type 1 diabetes influences the presence of PN with a U-shaped associative curve<sup>[23]</sup>. This may be true in the type 2 diabetic patient also<sup>[24]</sup>. There were a number of other comorbidities that were not associated with greater severity of PN in type 1 or type 2 diabetes patients. Peripheral neuropathy associated with MGUS<sup>[25]</sup> was rarely coincidental in DPN patients. Thyroid disorders (both hyperthyroidism<sup>[26]</sup> and hypothyroidism)<sup>[27]</sup>, frequently treated early after discovery, were not associated with further worsening of PN. Somewhat surprisingly, renal dysfunction, a potent cause of peripheral neuropathy<sup>[28]</sup> with potential relationship to diabetes<sup>[29]</sup> was not additive for DPN severity. Autoimmune disorders may have been too uncommon to contribute significantly to exacerbation of PN severity. Although speculated to impact upon DPN<sup>[3,30]</sup>, hypertension was not a significant contributor to DPN severity in our cohorts of type 1 or 2 diabetes patients. We did not examine other potential factors implicated in progression of DPN, such as smoking or body mass index due to incomplete data acquisition during assessments. It is possible that some of the above

comorbidities were not associated with greater severities of PN due to insufficient sample sizes.

We present these findings with limitations. Although we identified patients prospectively, they were not randomly selected from a population with type 1 or 2 diabetes with or without DPN; instead, these patients were referred for tertiary care. Our sample size was not based upon a pre-determined power analysis. We did not identify a separate cohort of patients with asymptomatic DPN. Higher HbA1C levels and longer durations of diabetes in patients with type 2 diabetes and comorbidities would certainly be anticipated to contribute to greater PN severity and may have impacted upon presented findings. Investigators were blinded to the laboratory results until clinical and electrophysiological studies were completed, but were not blinded to presence of type of diabetes or presence of comorbidities previously diagnosed. We did not use an established comorbidity burden tool to assess the studied comorbidities studied. All of our results were based upon TCSS and UENS scores—these are clinically relevant scales easily performed at the bedside, but have subjective components, and may not have the sensitivity of epidermal nerve fiber densities with skin biopsy<sup>[31]</sup> or confocal corneal microscopy<sup>[32]</sup>. Patients were on numerous medications for diabetes and other conditions; the heterogeneity of these medications and the conditions they were intended to treat made their individual assessment for contribution impossible. As a result, we did not exclude patients based upon any medication used, and acknowledge that this may have impacted upon clinical and electrophysiological assessments. Finally, this study was conducted at a single centre by a single assessor which could introduce biases due to referral patterns and assessment protocols used.

This study identified potential contributing comorbidities in approximately 30% of patients with diabetes and PN. Although we identified two potentially treatable specific comorbidities (cobalamin deficiency and lipid disorder), we do not yet know if management of these conditions will slow progression of PN, as may occur with procedures such as pancreatic islet transplantation<sup>[33]</sup> for treatment of type 1 diabetes. However, the use of simple, routine laboratory testing by primary physicians can identify factors for potential intervention in the future for DPN patients which may prevent clinical progression of PN. Lastly, the identification of these comorbidities, should not be viewed as a replacement for symptomatic relief, but as a potentially identifiable and modifiable component of an already diagnosed PN in patients with diabetes.

## COMMENTS

### Background

The pathophysiology of diabetic peripheral neuropathy remains uncertain and complex. Studies of comorbid conditions capable of causing peripheral neuropathy may assist in determination of causation of diabetic peripheral neuropathy and assist the clinician in managing patients with multiple conditions capable of causing peripheral neuropathy.

### Research frontiers

The presence of multiple potentially causative conditions is not uncommon in patients with diabetic peripheral neuropathy. Those comorbid conditions capable of worsening diabetic peripheral neuropathy may be subject to intervention, slowing the progression of peripheral neuropathy in patients with diabetes mellitus. Future studies should address the management of lipid disorders and vitamin B12 deficiency in populations with diabetic peripheral neuropathy.

### Innovations and breakthroughs

Although its isolated relationship to peripheral neuropathy is controversial, a lipid disorder was associated with greater severity of peripheral neuropathy in our patient populations with type 1 or 2 diabetes mellitus. However, lipidemia may have an additive effect when present with hyperglycemia present in diabetes mellitus. Also, prior studies have shown that metformin therapy is associated with impairment of vitamin B12 levels, a condition also associated with peripheral neuropathy, its management in patients with type 2 diabetes may also slow progression of diabetic peripheral neuropathy.

### Applications

Greater vigilance for other comorbidities in patients with diabetic peripheral neuropathy may reveal potentially manageable conditions that may be contributing to worsening of peripheral neuropathy over time.

### Peer review

The authors examined the severity of peripheral neuropathy in patients with diabetes mellitus with and without comorbid conditions capable of causing peripheral neuropathy. In particular, two conditions (lipid disorder and vitamin B12 deficiency) were detected and associated with greater neuropathic deficit. The results suggest that greater vigilance for these conditions may help patients with diabetic peripheral neuropathy by slowing the process of peripheral neurodegeneration.

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## Diabetes-related impairment in bone strength is established early in the life course

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### Abstract

**AIM:** To evaluate properties of bone quantity/quality using young non-obese Type 1 (T1D)-diabetic (NOD) prone and syngenic non-diabetic (NOD.*scid*) mice.

**METHODS:** Quantitative bone assessment of tibia was conducted using dual-energy X-ray absorptiometry (DXA) for the evaluation of body mass, bone mineral content, body fat mass and lean mass. Qualitative assessment was accomplished by three-point breakage for assessment of force to failure and micro-computed tomography for evaluation of trabecular and cortical properties of bone. In addition, fasting blood was

evaluated prior to sacrifice at week eleven and fifteen to evaluate and compare glucose homeostasis between the strains of mice.

**RESULTS:** Our findings support a perturbation in the relationship between bone quantity, quality, and subsequently, the association between structure and strength. There were no differences in DXA-assessed body composition (body fat, % fat mass and lean mass) and bone composition (bone mineral content and bone mineral density) between strains. However, relative to NOD.*scid*, NOD mice had lower trabecular bone volume, relative trabecular bone volume, trabecular number and trabecular total material density ( $P < 0.05$ ). Conversely, NOD mice had greater cortical total mean volume ( $P < 0.05$ ). General linear models analysis adjusted for body weight revealed a significant contribution of T1D to bone health as early as 5 wk.

**CONCLUSION:** It is well-established that diabetes is a significant risk factor for increased fractures, although the underlying mechanisms are not fully understood. Investigation of bone parameters encompassing strength and structure early in the life course will facilitate the elucidation of the pathogenesis of impaired bone integrity.

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**Key words:** Diabetes; Bone strength; Skeletal integrity; Development and growth; Non-obese diabetic

**Core tip:** Diabetes-related impairment in bone micro-architectural properties and parameters of quality was apparent as early as 5 wk.

Casazza K, Hanks LJ, Clines GA, Tse HM, Eberhardt AW. Diabetes-related impairment in bone strength is established early in the life course. *World J Diabetes* 2013; 4(4): 145-150 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i4/145>.

## INTRODUCTION

Apart from the classical complications of diabetes, adverse effects associated with bone health are becoming increasingly apparent. Individuals with diabetes have higher incidence of fracture and greater prevalence of osteoporosis. Many<sup>[1,2]</sup>, but not all<sup>[3-7]</sup> investigations report low bone mineral content (BMC) and density (BMD). Fracture healing is also compromised in diabetics with as high as 87% recovery delay relative to “healthy” counterparts<sup>[8-10]</sup>. While the skeletal manifestations of dysregulated glucose metabolism have been primarily considered in terms of bone quantity (*i.e.*, low bone mass), bone strength, the most obvious characteristic of bone structure/health is dependent upon various qualitative aspects.

A variety of animal models have been developed and used to examine the mechanisms of diabetes-related complications. Autoimmune-prone non-obese diabetic (NOD) mice are a widely studied model of spontaneous type 1 diabetes (T1D)<sup>[11-13]</sup>. In contrast to the pharmacologic streptozotocin (STZ)-induced T1D model, NOD mice become spontaneously diabetic secondary to a progressive diminished capacity of insulin-producing pancreatic beta islet cell function due to autoimmune destruction of the islet beta-cells. The earliest signs of autoimmune pathology in the NOD mouse occur at approximately 4 to 5 wk of age with leukocytes beginning to accumulate around the pancreatic islets, progressively intensifying and eventually leading to destruction of the insulin-producing beta cells at about 12 wk of age<sup>[14]</sup>. Whereas some studies have investigated bone phenotypes in adult NOD mice, the skeletal effects at disease initiation, to our knowledge have not been investigated. As a comparator strain, syngenic autoimmune-deficient NOD.*scid* mice lack functional lymphocytes, precluding the autoimmune destruction of beta cells and unlike NOD mice, do not develop T1D<sup>[11,13-16]</sup>.

While insulin stimulates not only osteoblastic cell differentiation, but also osteoblastogenesis and thus plays a pivotal role in bone metabolism<sup>[17,18]</sup>, an impairment in insulin regulation compromises bone processes giving rise to an altered phenotype. Accordingly, disease states related to insulin homeostasis/glucose handling might be expected to elicit profound physiologic alterations, particularly during growth<sup>[19]</sup>. The objective of the study was to evaluate properties of bone quantity (*via* DXA) and quality (*via* microCT, three point breakage) using young NOD *vs* NOD.*scid* mice, two different models in terms of insulin response to glucose. We hypothesized that as diabetes progressed in the NOD mice, the strength-structure impairments would manifest as illustrated by decreased force required to break, as well as trabecular and cortical bone measures.

## MATERIALS AND METHODS

### Mice

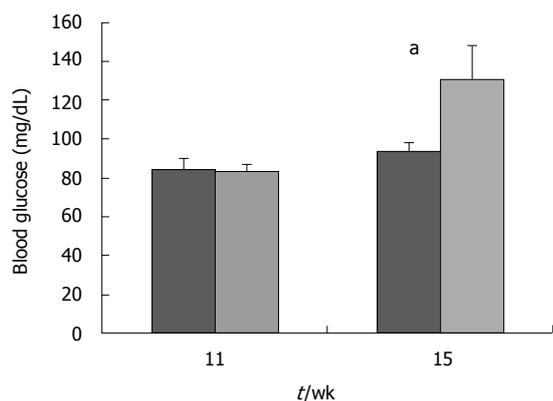
Five-to-seven week-old female NOD ( $n = 24$ ) and NOD.*scid* mice ( $n = 23$ ) were bred and housed at the Research Support Building of the University of Alabama at Birmingham, under pathogen-free conditions and observing IA-CUC approved mouse protocols. Mice were kept under a normal diurnal cycle in a temperature-controlled room and were fed with standard chow (NIH316 formula, Purina item #5K52) from age of weaning.

### Dual-energy X-ray absorptiometry

For assessment of bone quantity by dual-energy X-ray absorptiometry (DXA) analysis, animals were briefly anesthetized with isoflurane (2%) and placed in a prone position on the imaging plate. BMD, bone mineral content (BMC) was assessed *in vivo* (GE-Lunar PIXImus, software version 1.45, GE-Lunar). Additionally, lean mass and fat mass as well as animal area was obtained at 5, 8, 11 and 15 wk.

### Three-point breakage analysis and micro-computed tomography

For qualitative assessment, three point breakage and micro-computed tomography was used. Tibia strength was assessed by three-point breakage analysis using an MTS 858 MiniBionix (MTS Systems, Eden Prairie, MN) with a 100 N load cell. The span was 9 mm and the bones were loaded at a rate of 0.1 mm/s. For the determination of the 3D architecture of the trabecular and cortical bone, mouse femurs were scanned using the Scanco  $\mu$ CT40 desktop cone-beam micro-CT scanner (Scanco Medical AG, Brüttisellen, Switzerland). Femurs were placed vertically, but inverted (distal femur at the top) in 12  $\mu$ m diameter scanning holders and scanned twice: one for cortical and one for trabecular bone. Scans were performed at the following settings: 12 mm resolution, 55 kVp, 145  $\mu$ A with an integration time of 200 ms. Scans were automatically reconstructed into 2-D slices, and the region of interest was outlined in each slice using the micro-computed tomography ( $\mu$ CT) Evaluation Program (v5.0A, Scanco Medical). Cortical bone was determined at the mid-shaft of the femur with a scan of 25 slices. The region of interest (ROI) tool was used to outline the outside edge of the cortical bone. Cortical bone was identified and separated from the marrow by using a threshold value of 294. A 3D reconstruction was performed on the ROI consisting of everything within the outer cortical surface. Data was obtained for bone volume, (BV), bone density, total volume (TV) (bone plus marrow), bone volume fraction (BV/TV), trabecular thickness (Tb. Tk.), number (Tb. N) and cortical thickness. The scan of the trabecular bone was performed at the distal femur below the growth plate (on the inverted bone). Each scan consisted of 209 slices of which 100 were used for the analysis. ROI's were drawn on each of the 100 slices just inside



**Figure 1** Comparison of blood glucose (mg/dL) in non-obese diabetic (dark gray bars) and syngenic non-diabetic (light gray bars) mice at 11 and 15 wk demonstrates initiation of the type 1 diabetic phenotype. Error bars represent SEM. At five, eight (data not shown) and eleven there was no difference in blood glucose levels between strains of mice. <sup>a</sup>However at 15 wk, non-obese diabetic mice had significantly greater blood glucose than NOD.scid mice and concentration was indicative of diabetes (< 120 mg/dL).

the cortical bone, to include only the trabecular bone and marrow. Trabecular bone threshold was set at 226 HU, to distinguish it from the marrow. The 3D reconstruction was performed on the ROI which only contained trabecular bone; no cortical bone was present in these ROI's.

### Statistical analysis

Analysis of variance (ANOVA) was used to determine differences in bone properties between strains. If an aging effect was apparent for a given parameter, a post hoc comparison was performed. Statistical significance was set at  $\alpha < 0.05$ . In order to determine whether the strength-structure relationship was different, general linear models in which group, weight and body area were the covariates were conducted. All analyses were conducted using SAS (Institute Inc., Cary NC).

## RESULTS

Serum glucose was collected from 11 and 15 wk old NOD and NOD.scid mice. As expected, NOD mice displayed increased glucose beginning at 15 wk (Figure 1) and is consistent with the phenotype of this autoimmune T1D mouse model<sup>11-13</sup>. The growth characteristics were then determined between NOD and NOD.scid mice. At 5 wk, NOD mice were smaller than NOD.scid as represented by significantly less total mass, lean mass, body weight. However, by 8 wk size and weight did not differ and the similarity in body dimensions. At 15 wk NOD mice were significantly heavier (Figure 2).

We next analyzed the bone mechanical properties of tibiae harvested from 5 and 8 wk old NOD and NOD.scid mice. In a three-point bending test, NOD mice demonstrated less mechanical strength than NOD.scid mice at 5 wk but not at 8 wk of age (Figure 3).

Body composition and bone parameters of 5 and 8 wk old NOD and NOD.scid mice were next examined (Table 1). The weight of the NOD and NOD.scid mice

**Table 1** Pooled data from all mice ages 5 to 15 wk

Parameter	mean $\pm$ SEM NOD	NOD.scid
Body composition properties (DXA)		
Body weight	19.7 $\pm$ 0.6	19.9 $\pm$ 0.4
BMC	0.37 $\pm$ 0.40	0.34 $\pm$ 0.30
BMD	0.04 $\pm$ 0.04	0.040 $\pm$ 0.001
Body fat	2.8 $\pm$ 0.1	2.9 $\pm$ 0.2
Lean mass	14.7 $\pm$ 0.4	14.8 $\pm$ 0.2
% Fat	15.8 $\pm$ 0.7	16.2 $\pm$ 0.7
Trabecular bone properties (microCT)		
TV	1.5 $\pm$ 0.1	1.5 $\pm$ 0.1
BV	0.10 $\pm$ 0.01	0.15 $\pm$ 0.01 <sup>1</sup>
BV/TV	0.06 $\pm$ 0.01	0.10 $\pm$ 0.01 <sup>1</sup>
Tb. Th	0.050 $\pm$ 0.002	0.050 $\pm$ 0.002
Tb. N	2.3 $\pm$ 0.2	2.80 $\pm$ 0.11
Tb. MBV	931.9 $\pm$ 20.1	922.9 $\pm$ 20.7
Tb. TMD	92.2 $\pm$ 8.8	124.5 $\pm$ 9.7 <sup>1</sup>
Cortical bone properties (microCT)		
TV	0.19 $\pm$ 0.10	0.21 $\pm$ 0.01
BV	0.12 $\pm$ 0.02	0.120 $\pm$ 0.004
BV/TV	0.60 $\pm$ 0.01	0.60 $\pm$ 0.01
Ct. TMV	801.8 $\pm$ 22.5	770.4 $\pm$ 17.5 <sup>1</sup>
Ct. TMD	1288.5 $\pm$ 15.9	1291.6 $\pm$ 16.3

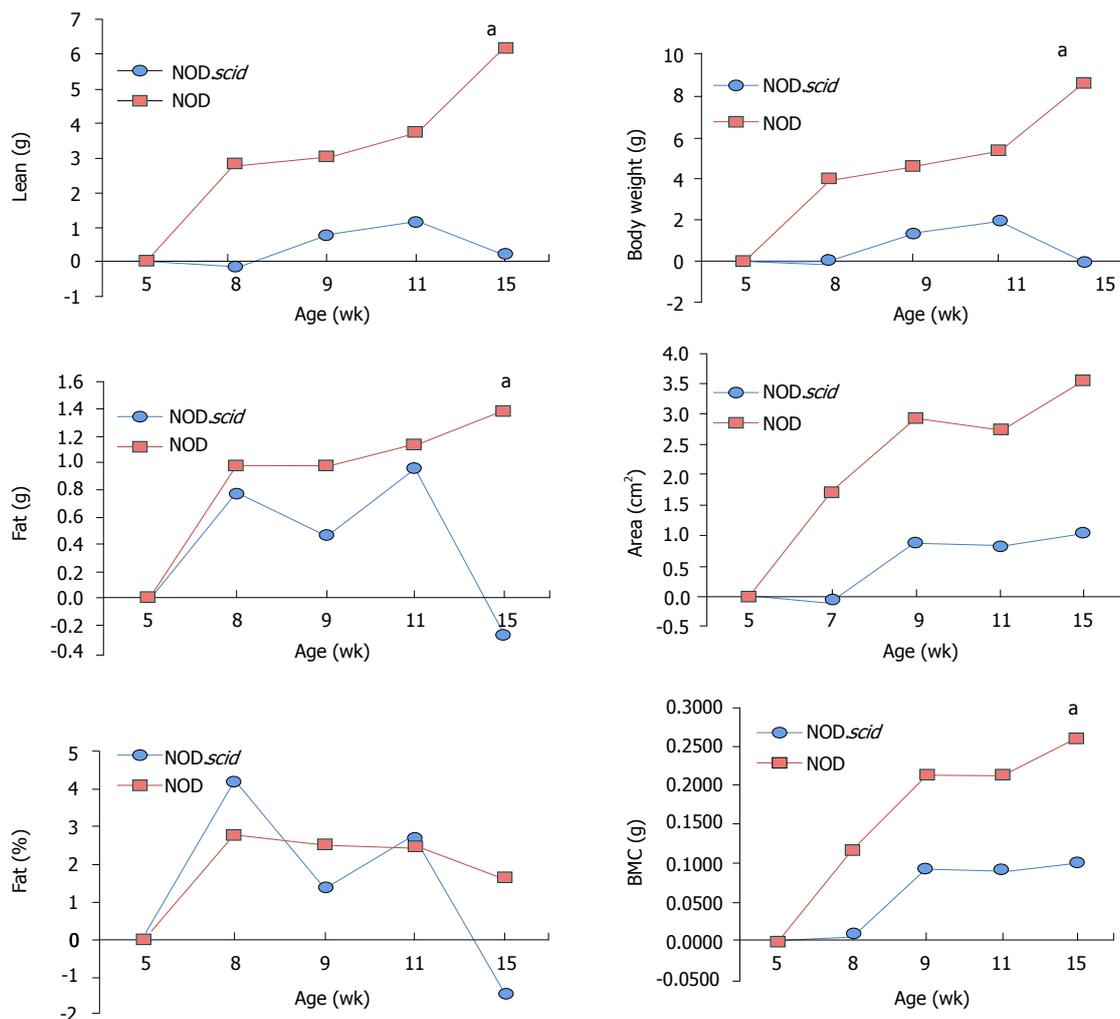
<sup>1</sup>Indicates significant difference between groups  $P < 0.05$ . BMC: Bone mineral content; BMD: Bone mineral density; BV: Bone volume; TV: Total volume (bone plus marrow); BV/TV: Bone volume fraction; Tb. Tk.: Trabecular thickness; Tb. N: Trabecular number; Tb. TMV: Trabecular material volume; Tb. TMD: Trabecular material density; Ct. TMV: Total cortical material volume; Ct. TMD: Total cortical material density; NOD: Non-obese diabetic; NOD.scid: Syngenic non-diabetic.

were similar (Table 1). There were no differences in body composition (body fat, fat mass and lean mass) and bone composition (bone mineral content and bone mineral density) were detected properties in NOD compared to NOD.scid mice (Table 1).

Because DXA can be an insensitive bone research technique, we performed microCT analyses to dissect the differences in trabecular and cortical bone. Tibiae were harvested from 5 and 8 wk old NOD and NOD.scid mice. Including all mice, there was not a significant difference in bending strength between NOD and NOD.scid mice. However, relative to NOD.scid, NOD mice had lower trabecular bone volume, relative trabecular bone volume, Tb.N, and trabecular total material density ( $P < 0.05$ ) (Table 1). Conversely, NOD mice had greater cortical total mean volume ( $P < 0.05$ ). We next performed a general linear models analysis that adjusted for body weight that revealed a significant contribution of age (Table 2). Accordingly, analysis was conducted by age. Table 2 presents the bone quality measures using microCT assessed at 5 and 8 wk. Diabetes-related impairment in bone microarchitectural properties and parameters of quality was apparent as early as 5 wk.

## DISCUSSION

There is a paucity of mechanistic information on how disease initiation and progression affect bone. While low bone mass in diabetes is often reported, material proper-



**Figure 2** Comparison of changes in body composition parameters as assessed by dual-energy X-ray absorptiometry at 5, 8, 11 and 15 wk. <sup>a</sup>Non-obese diabetic mice were significantly heavier.

ties of bone, specifically those addressing initiation of impairment in material properties is lacking. The present study provides support of impaired bone structure/architecture with diabetes on bone *via* impairment of bone structure/architecture early in the life course.

Pre-diabetic NOD mice had lower trabecular properties, but greater cortical volume, suggesting compositional differences exist in tissue properties prior to disease progression. Nyman and colleagues recently reported a time-dependent alteration in matrix organization beginning approximately ten weeks after STZ injection, in line with consistent reports<sup>[20]</sup>. The mice in the study were 11 wk old when injected. While the authors noted a decrement in mineralization, they subsequently observed an increase in non-enzymatic collagen cross-linking<sup>[21]</sup>. It is possible that injections in close proximity or prior to rapid skeletal growth may lead to accelerated change in the strength-structure relationship. Further, a later-induced diabetic phenotype provokes skeletal phenotypes *via* different pathways and several conditions in both models may have indirect effects on the reported properties of bone. Notably, beyond those initiated with diabetic onset

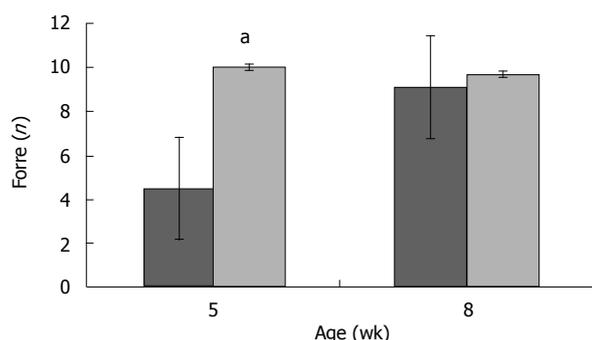
further changes in strength-structure relationship were not observed by Nyman and colleagues<sup>[20]</sup>. The increased Ct.MTV and Ct.TMD in NOD mice at 8 wk which did not translate into increased mechanical strength was surprising. Speculatively, a compensatory increase in insulin early in T1D prior to insulinitis may enhance anabolic properties at the outer surface. However, assessment of the strength-structure relationship requires evaluation of both outer and inner surfaces as well as the intrinsic properties within the bone (Ego Seeman, personal communication).

Particularly relevant during rapid skeletal growth, insulin has direct anabolic effects on periosteal apposition<sup>[17,18,21]</sup>. This would explain why diabetes did not affect the BMC or BMD, despite lower trabecular microarchitecture. In the context of humans, while it was recently reported that as adolescents with T1D attained reproductive maturation, had “normal” cortical cross sectional area<sup>[19]</sup>, fracture risk remains greater among this population. It is important to note that it was recently reported that while mechanical properties of bone in humans with diabetes were impaired relative to non-diabetic controls, strength was not differ-

**Table 2** Presents the bone quality measures using micro computed tomography assessed at 5 and 8 wk

	5 wk		8 wk	
	NOD (n = 4)	NOD.scid (n = 4)	NOD (n = 6)	NOD.scid (n = 4)
Body weight	14.9 ± 0.2	19.2 ± 0.4 <sup>1</sup>	19.2 ± 0.5	19.9 ± 0.6
BMC	0.19 ± 0.01	0.30 ± 0.01 <sup>1</sup>	0.30 ± 0.02	0.30 ± 0.01
BMD	0.030 ± 0.001	0.040 ± 0.002	0.040 ± 0.001	0.040 ± 0.001
Tibia strength	4.5 ± 0.2	10.0 ± 0.8	8.4 ± 0.7	8.2 ± 0.8
Trabecular bone properties				
TV	1.4 ± 0.2	1.6 ± 0.1	1.50 ± 0.06	1.40 ± 0.04
BV	0.08 ± 0.02	0.14 ± 0.01	0.11 ± 0.02	0.16 ± 0.05
BV/TV	0.05 ± 0.02 <sup>1</sup>	0.09 ± 0.00	0.07 ± 0.01	0.11 ± 0.04 <sup>1</sup>
Tb. Tk	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01
Tb. N	2.0 ± 0.3 <sup>1</sup>	2.8 ± 0.3	2.40 ± 0.09	2.90 ± 0.58
Tb. TMV	63.8 ± 4.9	108.9 ± 7.4 <sup>1</sup>	104.3 ± 8.9	137.0 ± 14.6
Tb. TMD	872.7 ± 42.5	930.5 ± 17.6	891.8 ± 21.3	948.8 ± 65.2
Cortical bone properties				
TV	0.18 ± 0.02 <sup>1</sup>	0.23 ± 0.02	0.20 ± 0.02	0.20 ± 0.02
BV	0.10 ± 0.02	0.14 ± 0.02	0.12 ± 0.00	0.11 ± 0.01
BV/TV	0.60 ± 0.03	0.60 ± 0.01	0.63 ± 0.01 <sup>1</sup>	0.58 ± 0.01
Ct. TMV	743.5 ± 56.9	787.4 ± 21.5	831.0 ± 13.2	753.0 ± 16.2 <sup>1</sup>
Ct. TMD	1262.9 ± 18.9	1324.1 ± 20.8	1304.3 ± 11.0	1258.6 ± 13.4

<sup>1</sup>Indicates significant difference between groups  $P < 0.05$ . BMC: Bone mineral content; BMD: Bone mineral density; BV: Bone volume; TV: Total volume (bone plus marrow); BV/TV: Bone volume fraction; Tb. Tk: Trabecular thickness; Tb. N: Trabecular number; Tb. TMV: Trabecular material volume; Tb. TMD: Trabecular material density; Ct. TMV: Total cortical material volume; Ct. TMD: Total cortical material density; NOD: Non-obese diabetic; NOD.scid: Syngenic non-diabetic.



**Figure 3** Tibia strength by 3-point breakage analysis using the NTS 85MTS Minibionix8 with a 100 N load cell. The span was 9 mm and the bones were loaded with a rate of 0.1 mm/s to evaluate maximum load to failure in 5 and 8 wk in non-obese diabetic (dark gray bars) and syngenic non-diabetic (light gray bars). Error bars represent SEM. <sup>a</sup>Non-obese diabetic mice demonstrated less mechanical strength than syngenic non-diabetic mice at 5 wk.

ent between middle-aged and older adults with diabetes<sup>[22]</sup>, supporting our findings of the deleterious effects on bone integrity initiated early in disease progression.

While NOD mice are used to examine spontaneous T1D progression, immune-deficient NOD.scid mice may incur indirect effects on bone properties, which may explain the reported differences in the literature. Lacking mature B and T cells, NOD.scid mice are both insulinitis- and diabetes-free throughout life. However, because of a high incidence of thymic lymphomas, the mean lifespan is relatively short<sup>[13,23,24]</sup>. Accordingly, while the unique immune defects provide an excellent *in vivo* environment for hematopoietic investigation extending to effects within the marrow compartment, this model may not be suitable for assessing bone material properties. The long-term

tumorigenic effects provoked an unanticipated effect on growth in NOD.scid mice that likely affected strength-structure relationship. Further investigation in a comparable strain with optimal growth conditions [*e.g.*, non-obese diabetes resistant (NOR)] are needed to confirm our findings.

In conclusion, the T1D mouse model revealed complex changes early in the developmental process. There was diminished trabecular microarchitecture which manifested into weakened bone strength relative to non-diabetic mice, independent of bone mass. Our findings support a perturbation in the relationship between bone structure and strength and a need for intervention efforts to promote bone parameters during growth and development.

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## COMMENTS

### Background

While the skeletal manifestations of dysregulated glucose metabolism have been primarily considered in terms of bone quantity (*i.e.*, low bone mass), bone strength, the most obvious characteristic of bone structure/health is dependent upon various qualitative aspects.

### Research frontiers

Diabetes-related impairments in insulin/glucose handling alter growth processes including those associated with bone development. However, the precise mechanisms accounting for perturbed bone accretion processes are not known and may differ, at least in part, by degree of glucose control.

### Innovations and breakthroughs

Maintenance of glucose in circulation within a “normal” range, as well as the standard of “normality” throughout dynamic growth processes, varies, and has particular relevance to body composition trajectories during critical periods of development.

### Applications

Peak bone mass, a major determinant of adult bone health is largely achieved by the end of sexual and skeletal maturity. Thus, an emerging area of investigation is the contribution of insulin/glucose homeostasis to bone (re)modeling, with considerable interest in understanding influential factors serving to maximize bone mass accrual in childhood, and therefore optimize bone phenotype throughout life.

### Peer review

A potential explanation for observed differences in bone parameters in adults may be related to impairment in the remodeling-associated bone-resorption/formation coupling, which is maximally operational during the rapid skeletal development phase in childhood. Although the process of bone remodeling is complex, accumulating evidence supports glucose homeostasis as an integral part of bone formation (quantity) and micro-architecture (quality). Consistent with this, individuals exhibiting impaired glucose handling are at risk for compromised bone mass and integrity. In particular, type 1 diabetes mellitus is associated with increased bone mass loss (*e.g.*, osteopenia, osteoporosis), increased risk of fragility fracture, and poor bone healing following injury. Investigation early in the life course is highly relevant.

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## Vildagliptin-insulin combination improves glycemic control in Asians with type 2 diabetes

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### Abstract

**AIM:** To assess the efficacy and safety of vildagliptin 50 mg *bid* as add-on therapy to insulin in Asian patients with type 2 diabetes mellitus (T2DM).

**METHODS:** This was a post hoc analysis of a subgroup of Asian patients from a multicenter, randomized, double-blind, placebo-controlled, parallel-group study in T2DM patients inadequately controlled by stable insulin therapy, with or without metformin. A total of 173 patients were randomized 1:1 to receive treatment with vildagliptin 50 mg *bid* ( $n = 87$ ) or placebo ( $n = 86$ ) for 24 wk. Changes in HbA1c and fasting plasma glucose (FPG), from baseline to study endpoint, were analyzed using an analysis of covariance model. Change from baseline to endpoint in body weight was

summarized by treatment. Safety and tolerability of vildagliptin was also evaluated.

**RESULTS:** After 24 wk, the difference in adjusted mean change in HbA1c between vildagliptin and placebo was 0.82% (8.96 mmol/mol;  $P < 0.001$ ) in Asian subgroup, 0.85% (9.29 mmol/mol;  $P < 0.001$ ) in patients also receiving metformin, and 0.73% (7.98 mmol/mol;  $P < 0.001$ ) in patients without metformin, all in favor of vildagliptin. There was no significant difference in the change in FPG between treatments. Weight was stable in both treatment groups (+0.3 kg and -0.2 kg, for vildagliptin and placebo, respectively). Overall, vildagliptin was safe and well tolerated with similarly low incidences of hypoglycemia (8.0% vs 8.1%) and no severe hypoglycemic events were experienced in either group.

**CONCLUSION:** In Asian patients inadequately controlled with insulin (with or without concomitant metformin), insulin-vildagliptin combination treatment significantly reduced HbA1c compared with placebo, without an increase in risk of hypoglycemia or weight gain.

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**Key words:** Asian; DPP-4 inhibitor; Hypoglycemia; Insulin; Oral antidiabetic drug; Type 2 diabetes; Vildagliptin

**Core tip:** In Asian patients, vildagliptin added to stable dose of insulin, with or without concomitant metformin, significantly improves glycemic control without increase in weight and hypoglycemia incidence.

Kozlovski P, Foley J, Shao Q, Lukashevich V, Kothny W. Vildagliptin-insulin combination improves glycemic control in Asians with type 2 diabetes. *World J Diabetes* 2013; 4(4): 151-156 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i4/151.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i4.151>

## INTRODUCTION

The unfolding diabetes epidemic is projected to affect more than 550 million people worldwide by the year 2030 with approximately 60% of patients coming from Asia<sup>[1]</sup>. Despite available antidiabetic treatments, glycemic control in most Asian countries is unsatisfactory<sup>[2,3]</sup>. The progressive nature of type 2 diabetes requires continuous treatment intensification with a combination of antidiabetic agents having different mechanisms of action, and initiation of insulin therapy when beta cell function significantly deteriorates. However, delay in insulin initiation and intensification is a major problem across the world. In Asia, the mean HbA1c at the time of insulin intensification exceeds 9%<sup>[4]</sup>, with fear of hypoglycemia and concern of weight gain identified as the main barriers for early and optimal insulin use<sup>[5]</sup>. Therefore, antidiabetic agents that can significantly improve glycemic control without increasing the risk of hypoglycemia and weight gain when used in combination with insulin are needed. While the use of insulin in combination with oral antidiabetic drugs (OADs) is increasing in Asia<sup>[5]</sup>, there is little data from randomized controlled trials investigating the efficacy and safety of OAD-insulin combination in Asian patients with type 2 diabetes mellitus (T2DM).

Vildagliptin is a potent and selective inhibitor of dipeptidyl peptidase-4 (DPP-4), which extends the physiological effects of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) resulting in improvement of glycemic control in a glucose-sensitive manner<sup>[6-8]</sup>. In Asian patients with T2DM, vildagliptin showed significant improvements in HbA1c with low incidence of hypoglycemia when used as monotherapy<sup>[9]</sup>, in combination with metformin<sup>[10]</sup>, or in combination with a sulfonylurea<sup>[11]</sup>.

We recently reported that vildagliptin added to insulin therapy resulted in a robust improvement in glycemic control without increasing the risks of hypoglycemia and weight gain<sup>[12]</sup>. This study included about 40% patients from Asia ( $n = 173$ ) allowing for a meaningful analysis of the efficacy and safety data in this population. Asian patients with T2DM could be more susceptible to hypoglycemia than Caucasians due to their lower body weight and increased sensitivity to insulin<sup>[13,14]</sup> and there is a general lack of data in Asians as discussed above. We, therefore, analyzed the subgroup of Asian patients in this study to characterize the response to vildagliptin when combined with insulin in this growing patient population.

## MATERIALS AND METHODS

### Study design and patients

This was a post hoc analysis of a subgroup of Asian patients from a 24 wk, multicenter, randomized, double-blind, placebo-controlled, parallel-group study. Eligible patients included 89 men and 84 women aged 18-80 years with T2DM, HbA1c  $\geq 7.5\%$  ( $\geq 58.5$  mmol/mol) and  $\leq 11.0\%$  ( $\leq 96.7$  mmol/mol), and fasting plasma glucose levels (FPG)  $< 15$  mmol/L, who were being treated with stable insulin doses  $\leq 1$  U/kg per day (long-acting, inter-

mediate-acting, or premixed, once or twice daily) with or without stable concomitant metformin treatment ( $\geq 1500$  mg or maximally tolerated dose) for at least 12 wk.

After a 2 wk screening period, patients were randomized in a 1:1 ratio to treatment with vildagliptin 50 mg *bid* or placebo. Randomization was stratified by metformin use and type of insulin used (long-acting *vs* intermediate acting/premixed). Further details of the study design were reported by Kothny *et al*<sup>[12]</sup>.

### Study assessments and endpoints

HbA1c, FPG, and body weight were assessed at every visit, scheduled at 4 wk intervals. The efficacy endpoints were the change in HbA1c, FPG, and body weight from baseline to 24 wk or to the final visit. Safety assessments included monitoring and recording of treatment emergent adverse events (AEs), serious AEs (SAEs), biochemistry and hematology laboratory test results, electrocardiogram (ECG) findings, and vital signs.

Hypoglycemia was defined by symptoms suggestive of hypoglycemia and a self-monitored plasma glucose measurement  $< 3.1$  mmol/L. Severe hypoglycemia was defined as an episode that required assistance of another person or hospitalization with or without a plasma glucose measurement  $< 3.1$  mmol/L.

### Statistical analysis

The changes in HbA1c from baseline to week 24 were compared between vildagliptin and placebo using an analysis of covariance with treatment, region, metformin use, and insulin type as classification variables and baseline HbA1c as covariate. This comparison was performed for the overall Asian population and for patients with/without concomitant metformin. Change in FPG was analyzed using the same model as for HbA1c. In addition, responder rates [percentage of patients achieving endpoint HbA1c  $< 7.0\%$  (53.0 mmol/mol)] were compared between treatments using a chi-squared test. The efficacy analyses were performed on the full analysis set population consisting of all randomized patients who received at least one dose of the study drug and had at least one post-baseline assessment of any efficacy variable.

Efficacy data used in analyses were censored at the start of major changes in insulin background therapy. Major changes in insulin therapy were defined as changes occurring  $\geq 7$  d in any 30-d period or  $\geq 5$  d consecutively, including changes in insulin frequency and/or type and/or a  $\geq 10\%$  dose increase either as rescue medication or for any other reasons. The last observation carried forward (LOCF) method was used to handle missing data because of early discontinuation or data censoring.

Change in body weight from baseline to endpoint was summarized descriptively. The safety data (AEs, SAEs, including hypoglycemia) were summarized descriptively by treatment on all available data.

### Ethical considerations

This trial was conducted in accordance with the Declara-

**Table 1** Baseline patient demographic and background characteristics (Asian population)

	Vildagliptin 50 mg <i>bid</i> <i>n</i> = 87	Placebo <i>n</i> = 86
Age, years	54.0 ± 8.4	54.9 ± 10.5
≥ 65, <i>n</i> (%)	8 (9.2)	13 (15.1)
Gender, female, <i>n</i> (%)	45 (51.7)	39 (45.3)
Race, <i>n</i> (%)		
Indian (Indian subcontinent)	62 (71.3)	61 (70.9)
Chinese	24 (27.6)	24 (27.9)
Other	1 (1.1)	1 (1.2)
BMI, kg/m <sup>2</sup>	26.2 ± 3.0	26.7 ± 3.7
Body weight, kg	67.5 ± 9.5	68.8 ± 12.1
HbA1c, % (mmol/mol)	8.9 ± 1.0 (73.7 ± 10.9)	9.0 ± 1.0 (74.8 ± 10.9)
FPG, mmol/L	9.1 ± 2.6	8.6 ± 2.5
T2DM duration, years	11.1 ± 6.4	12.1 ± 7.6
GFR, mL/min per 1.73 m <sup>2</sup> , <i>n</i> (%)		
Normal, > 80	43 (49.4)	52 (60.5)
Mild, ≥ 50 to ≤ 80	42 (48.3)	33 (38.4)
Moderate, ≥ 30 to < 50	2 (2.3)	1 (1.2)
Background antidiabetic therapy		
Insulin use at screening, <i>n</i> (%)		
Intermediate-acting	21 (24.1)	20 (23.3)
Long-acting	14 (16.1)	8 (9.3)
Pre-mixed	52 (59.8)	58 (67.4)
Duration of insulin use, years	3.3 ± 2.9	3.9 ± 4.3
Daily dose of insulin, U	39.5 ± 15.8	39.5 ± 15.3
Daily number of insulin injections	1.9 ± 0.4	1.8 ± 0.4
Metformin use at screening, <i>n</i> (%)		
Yes	52 (59.8)	52 (60.5)
No	35 (40.2)	34 (39.5)

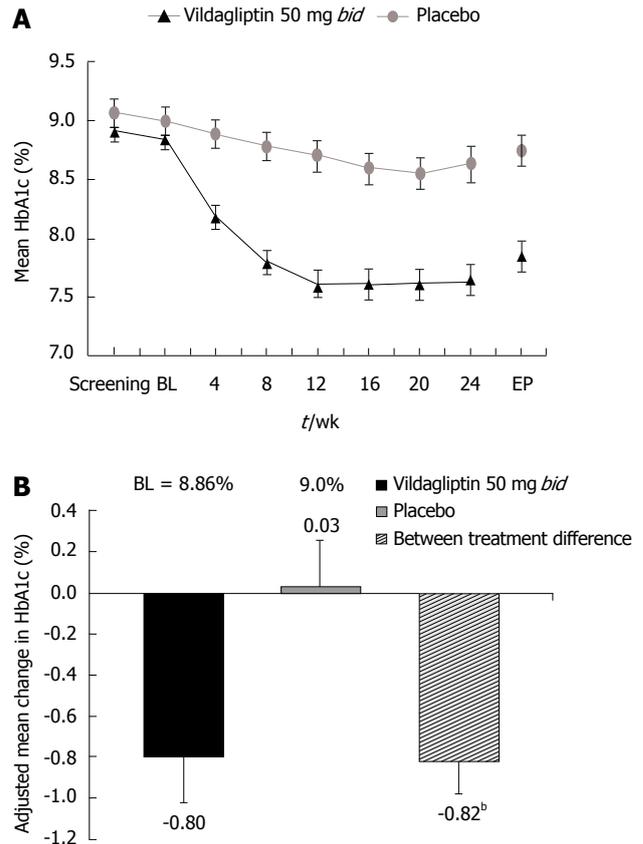
Values are mean ± SD unless indicated otherwise. BMI: Body mass index; GFR: Glomerular filtration rate; FPG: Fasting plasma glucose; HbA1c: Hemoglobin A1c; T2DM: Type 2 diabetes mellitus.

tion of Helsinki. An independent ethics committee or institutional review board at each research site reviewed the study protocol. Each patient gave written informed consent before randomization.

## RESULTS

### Patient disposition and baseline characteristics

A total of 173 Asian patients (38.5% of the overall study population) were randomized: 87 patients to vildagliptin 50 mg *bid* and 86 patients to placebo. The demographic and baseline characteristics of the randomized patients are summarized in Table 1. The groups were well balanced for all the baseline characteristics. Most patients were from the Indian subcontinent (71%) followed by patients of Chinese ethnicity (27.7%) with a mean age of 54.5 years; just over 12% of patients were ≥ 65 years of age. Mean baseline values of HbA1c and FPG were 8.9% (73.8 mmol/mol) and 8.8 mmol/L, respectively. Mean body mass index (BMI) was 26.4 kg/m<sup>2</sup> and the majority (86%) had BMI < 30 kg/m<sup>2</sup>. The mean duration of T2DM was 11.6 years. Mean duration of insulin usage was 3.6 years, mean daily insulin dose at screening was 39.5 units, and pre-mixed insulin was the most frequent type of insulin used. Overall, 60.1% of patients were treated with metformin. The mean metfor-



**Figure 1** Mean change. A: Mean change in HbA1c over time; B: Adjusted mean change in HbA1c from baseline to endpoint. BL: Baseline; EP: Endpoint; <sup>b</sup>*P* < 0.001.

min dose at the time of randomization was approximately 2000 mg for both treatment groups.

### Efficacy

In this Asian population, vildagliptin demonstrated consistent reductions in HbA1c from baseline to week 24 endpoint (Figure 1A). After 24 wk of treatment, HbA1c had decreased by 0.8 ± 0.2% (8.74 ± 2.2 mmol/mol) in patients receiving vildagliptin (*n* = 85) and HbA1c increased by 0.03 ± 0.2% (0.32 ± 2.2 mmol/mol) in patients receiving placebo (*n* = 84). The adjusted between-treatment difference (vildagliptin 50 mg *bid*-placebo) in HbA1c of 0.82 ± 0.1% (8.96 ± 1.1 mmol/mol) was statistically significant (*P* < 0.001) in favor of vildagliptin (Figure 1B).

Vildagliptin significantly reduced HbA1c in both patients with and without concomitant metformin therapy, with adjusted mean differences *vs* placebo of 0.85% (9.29 mmol/mol) and 0.73% (7.98 mmol/mol) (*P* < 0.001 for both groups), respectively, in favor of vildagliptin. In subgroups by ethnicity, reductions in HbA1c from baseline were 0.99% (10.82 mmol/mol) and 1.17% (12.78 mmol/mol) with vildagliptin, and 0.31% (3.38 mmol/mol) and 0.08% (0.87 mmol/mol) with placebo, in Indian and Chinese patients, respectively.

In a responder analysis, significantly more patients receiving vildagliptin achieved the HbA1c target of < 7.0%

(53.0 mmol/mol) than those receiving placebo (22.4% and 4.8%, respectively;  $P = 0.001$ ). FPG did not change significantly in the vildagliptin group ( $n = 85$ ) with a 0.2 mmol/L increase at week 24 from baseline of 9.6 mmol/L; a more pronounced change was seen in the placebo group ( $n = 84$ ) with a 0.7 mmol/L increase at week 24 from baseline of 9.0 mmol/L; mean placebo-subtracted difference was 0.5 mmol/L ( $P = 0.335$ ) in favor of vildagliptin.

The mean insulin dose at baseline was 39.5 units in both vildagliptin and placebo groups. The mean changes from baseline to study end were reductions of 1.39 and 1.48 units in the vildagliptin group and the placebo group, respectively. Overall, the small changes in the insulin dose in both treatment groups are consistent with the protocol requirement for a stable insulin dose during the study.

### Safety

Vildagliptin 50 mg *bid* added to intermediate-acting, long-acting, or premixed insulin, with or without metformin, was generally safe and well tolerated.

The overall incidence of AEs was numerically higher with vildagliptin (62.1%) than with placebo (53.5%). This difference was driven by gastrointestinal disorders (14.9% *vs* 7.0%), blurred vision (9.2% *vs* 0%), and upper respiratory tract infections (13.8% *vs* 8.1%). The latter were assessed by investigators as mild or moderate and not related to study drug. Diarrhea and gastritis were more frequently reported in the vildagliptin group; however, the drug was not discontinued in any of the cases.

The proportion of patients who experienced hypoglycemic events was low and similar in both treatment groups (8.0% and 8.1% in the vildagliptin and placebo groups, respectively). No patient in either treatment group experienced a severe hypoglycemic event. Similar number of patients in the vildagliptin and placebo groups reported hyperhidrosis, dizziness, tremors, and palpitations, which may be symptoms of hypoglycemia. Blurred vision was reported by 8 patients (9.2%). For three of them, blurred vision was identified as hypoglycemia and included in the hypoglycemic events summary since they had accompanying glucose measurements  $< 3.1$  mmol/L. Of the remaining five, four reported, together with the blurred vision, one or more other symptoms suggestive of hypoglycemia (dizziness, weakness, palpitations, tremor or hyperhidrosis); however no blood glucose measurement had been performed to confirm a hypoglycemic event. Six of these eight patients experienced considerable reduction in HbA1c of 1.4% (15.3 mmol/mol) or more during the study; another one had a smaller HbA1c reduction, but reached HbA1c of 6.5% (47.5 mmol/mol). These events of blurred vision could be symptoms of hypoglycemia, or in some cases a reflection of rapidly improving glucose levels.

The rate of serious AEs was very low in this subgroup with only one serious AE reported. This was a case of liver enzyme elevation reported in one vildagliptin-treated patient with history of non-alcoholic steatohepatitis. This event was associated with respiratory infection and the adjudication committee concluded that it was

unrelated to study drug.

Body weight remained stable during the study with an increase of 0.3 kg in the vildagliptin group and a decrease of 0.2 kg in the placebo group. Overall, the safety profile of vildagliptin in the Asian subgroup was consistent with the safety profile in the overall patient population<sup>[12]</sup>, without any clinically relevant differences between treatments.

## DISCUSSION

In Asian patients, the addition of vildagliptin 50 mg *bid* to stable therapy with basal or pre-mixed insulin, with or without concomitant metformin, demonstrated a robust reduction in HbA1c *vs* placebo after 24 wk of treatment. Vildagliptin was efficacious in patients both from Indian and Chinese ethnicity with clinically relevant reductions in HbA1c from baseline of about 1.0% (10.93 mmol/mol). Importantly, the addition of vildagliptin to insulin was not associated with an increased risk for hypoglycemia or weight gain. These findings are consistent with the results from the overall study population<sup>[12]</sup>. Mean baseline HbA1c was similar in both the overall population [8.8% (72.7 mmol/mol)] and in the Asian population [8.9% (73.8 mmol/mol)] and so was the reduction in HbA1c *vs* placebo after 24 wk of treatment [0.7% (7.6 mmol/mol) and 0.8% (8.7 mmol/mol), respectively].

Asian patients had lower BMI than patients in the overall study population (26.4 kg/m<sup>2</sup> *vs* 28.9 kg/m<sup>2</sup>, respectively) which could make them more sensitive to insulin and, thus, place them at a higher risk of hypoglycemia<sup>[15]</sup>. However, the incidence of hypoglycemia was similar for vildagliptin and placebo in spite of better glycemic control with vildagliptin indicating that vildagliptin exerts its protective effect against hypoglycemia also in Asian patients. Vildagliptin has demonstrated a protective effect against hypoglycemia at all stages of type 2 diabetes<sup>[16]</sup> resulting from its ability to increase glucagon levels during hypoglycemia<sup>[6]</sup>.

In this study, adding vildagliptin to a stable insulin dose was weight neutral, which is consistent with the known vildagliptin weight profile when used as monotherapy or in combination with other OADs<sup>[17-21]</sup>. The weight neutrality of vildagliptin likely results in part from its intrinsically low risk for hypoglycemia and avoidance of “defensive eating” characteristic for antidiabetic agents associated with increased hypoglycemia risk. Other potential mechanisms may include possible inhibition of intestinal fat extraction and fatty acid mobilization and oxidation in the postprandial state, in conjunction with increased sympathetic stimulation<sup>[21]</sup>.

Multinational studies with DPP-4 inhibitors added to insulin included small numbers of Asian patients<sup>[22-24]</sup> and, therefore, meaningful analysis might have not been possible. However, in a Korean study, addition of sitagliptin to a stable insulin dose resulted in reduction in HbA1c of 0.6% (6.5 mmol/mol) from baseline of 9.2% (77.1 mmol/mol), with one patient experiencing severe hypoglycemia<sup>[25]</sup>. This is consistent with the find-

ings from a multinational study with sitagliptin, which showed improvement in glycemic control at the expense of increased hypoglycemia incidence compared with placebo<sup>[24]</sup>. In contrast, addition of vildagliptin to insulin in this study was not associated with an increased risk of hypoglycemia in Asian patients and no events of severe hypoglycemia were reported. This difference between vildagliptin and sitagliptin could be due to vildagliptin's ability to maintain elevated GIP levels into periods where hypoglycemia is likely to occur, thus resulting in improved glucagon counter-regulation<sup>[26]</sup>.

In conclusion, the presented efficacy and safety data in Asian patients inadequately controlled with a stable insulin dose with or without concomitant metformin showed that vildagliptin can be a suitable add-on treatment leading to improved glycemic control without increased risk of hypoglycemia or weight gain. Despite some differences in diabetes phenotype between Asians and Caucasians as well as potential differences in the pathophysiology of T2DM in these populations, the beneficial effects of vildagliptin when added to insulin are maintained in an Asian population.

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## COMMENTS

### Background

The increasing diabetes epidemic by 2030 with majority of patients from Asia is of major concern. The progressive nature of disease requires intensified treatment with multiple antidiabetic agents, and insulin initiation when beta cell function deteriorates. Therefore, agents which improve glycemic control without hypoglycemia and weight gain when used with insulin are needed. However, there is little data from randomized controlled trials investigating the efficacy and safety of oral antidiabetic drugs-insulin combination in Asian patients with type 2 diabetes. The authors recently reported that vildagliptin added to insulin therapy resulted in a robust improvement in glycemic control without increasing the risks of hypoglycemia and weight gain. This study included about 40% patients from Asia and thus the authors analyzed the subgroup of Asian patients to characterize the response to vildagliptin when combined with insulin in a patient population in which diabetes is a growing concern.

### Research frontiers

Vildagliptin is a selective inhibitor of dipeptidyl peptidase-4 (DPP-4) enzyme, improves glycemic control by increasing the availability of incretins. Considering that Asian patients with type 2 diabetes could be more susceptible to hypoglycemia than Caucasians due to their lower body weight and increased sensitivity to insulin, and due to the general lack of data in Asians, the efficacy and safety of vildagliptin-insulin combination in this population was assessed.

### Innovations and breakthroughs

This is the first double-blind placebo controlled study that reports the efficacy and safety of a DPP-4 inhibitor (vildagliptin) as add-on to insulin in an Asian population.

### Applications

This study demonstrates that vildagliptin in combination with insulin is a safe and efficacious antidiabetic treatment by significantly reducing HbA1c without an increased incidence of hypoglycemia or weight gain.

### Terminology

DPP-4 inhibitors: Dipeptidyl peptidase-4 inhibitors are a class of oral antihyper-

glycemic agents that inhibit the enzyme DPP-4. They are used to treat type 2 diabetes mellitus. HbA1c: Glycated hemoglobin is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. It is formed in a non-enzymatic glycation pathway by hemoglobin's exposure to plasma glucose. The 2010 American Diabetes Association Standards of Medical Care in Diabetes added the HbA1c  $\geq$  48 mmol/mol ( $\geq$  6.5%) as another criterion for the diagnosis of diabetes.

### Peer review

This manuscript presents an analysis of the Asian subgroup of a recently published study. It addresses new findings regarding the effects of vildagliptin as add-on therapy to insulin in Asian patients with type 2 diabetes mellitus. The present work contains interesting data and appears timely.

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## Effect of treatment of overt hypothyroidism on insulin resistance

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### Abstract

**AIM:** To investigate the impact of hypothyroidism and thyroxine therapy on insulin sensitivity in patients with overt hypothyroidism.

**METHODS:** The study included twenty seven overtly hypothyroid and fifteen healthy euthyroid South Western Asian females. Both groups had matching age and body mass index. Physiological and pathological conditions as well as medications that may alter thyroid function, glucose homeostasis or serum lipids were ruled out. Serum thyrotropin (TSH), free tetraiodothyronine (FT4), free triiodothyronine (FT3), fasting insulin (FI), fasting plasma glucose (FPG), total cholesterol and triglycerides were measured before and six months after initiating thyroxine therapy for hypothyroid patients and once for the control group. Insulin resistance (IR) was estimated using homeostasis model assessment (HOMA-IR) and Body mass index (BMI) was calculated.

**RESULTS:** Both study groups, hypothyroid patients and euthyroid control subjects, had matching age and body mass index ( $P$ -value 0.444, 0.607 respectively). No significant difference was found between the hypothyroid patients and the euthyroid control group re-

garding fasting plasma glucose, fasting insulin, insulin resistance, total cholesterol and triglycerides ( $P$ -values 0.432, 0.621, 0.883, 0.586, 0.05 respectively). In the hypothyroid patients, triglycerides showed direct correlation to TSH and inverse correlation to FT3. Similarly total cholesterol inversely correlated to FT3 but its direct correlation to TSH did not reach statistical significance. After thyroxine replacement and reaching an euthyroid state as confirmed by clinical and laboratory data, there was no significant change in fasting plasma glucose, insulin resistance or triglyceride level ( $P$ -value 0.216, 0.204, 0.175 respectively) while total cholesterol significantly decreased ( $P$ -value 0.043) and fasting insulin significantly increased ( $P$ -value 0.047).

**CONCLUSION:** Hypothyroidism has no impact on insulin sensitivity. Correction of hypothyroidism is not associated with a significant change of insulin sensitivity or triglycerides, but with a significant reduction of total cholesterol.

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**Key words:** Hypothyroidism; Female; Thyroxine; Insulin resistance; Triglycerides; Cholesterol

**Core tip:** Thyroid dysfunction is the second most common endocrine disorder after diabetes mellitus. Both diseases are strong associated. Hypothyroidism is claimed to cause insulin resistance. Some available reports are in agreement and others are against this suggestion. In our study, we did not find a significant effect of hypothyroidism or thyroxine replacement on insulin resistance as calculated by insulin resistance was estimated using homeostasis model assessment. Thyroxine therapy leads to a significant reduction of total cholesterol but it does not change triglycerides. This may partially explain the association between hypothyroidism and dyslipidaemia as well as cardiovascular risk.

Nada AM, Effect of treatment of overt hypothyroidism on insulin resistance. *World J Diabetes* 2013; 4(4): 157-161 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i4/157.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i4.157>

## INTRODUCTION

Thyroid dysfunction and diabetes mellitus (DM) are the two most common endocrine disorders. Both disorders appear to be closely linked<sup>[1]</sup>. A recent meta-analysis that was conducted on available data in 10920 patients with DM revealed a mean frequency of thyroid disease of 11% with no difference between type 1 DM and type 2 DM. The prevalence in women was consistently more than two-folds that in men<sup>[2]</sup>.

It has also been postulated that insulin secretion is regulated by the thyroid hormone<sup>[3,4]</sup> and diabetes risk is related to thyroid hormone levels<sup>[5,6]</sup>. The initial event of glucose-stimulated insulin secretion is glucose sensing. The glucose transporter 2 (GLUT2) and glucokinase (GK) are key molecules which affect various processes of glucose sensing in pancreatic  $\beta$ -cells<sup>[7]</sup>. Impairment in glucose sensing contributes to pancreatic  $\beta$ -cell dysfunction. Therefore, it is necessary to maintain adequate expression levels of GLUT2 and GK to ensure normal  $\beta$ -cell function<sup>[8]</sup>. Triiodothyronine (T<sub>3</sub>) can modulate the expression of GLUT2 and GK mRNAs and proteins in pancreatic islets<sup>[9]</sup> and liver<sup>[10]</sup>.

To date, only a few studies have investigated the effect of hypothyroidism and its recovery by thyroid hormone treatment on glucose metabolism and lipid profile, and the results have been controversial. Some researchers elucidated lower insulin sensitivity in patients with overt hypothyroidism which improved after thyroxine treatment<sup>[11,12]</sup>. Subclinical hypothyroidism was also encountered as a cause of insulin resistance and its related dyslipidaemia in patients with rheumatoid arthritis<sup>[13]</sup>. Contrary to that, Brenta *et al*<sup>[14]</sup> did not find significant differences in insulin sensitivity or lipid profile before and after thyroxine replacement in subclinical hypothyroidism.

In the light of existing data, we decided to study the impact of hypothyroidism on insulin sensitivity in overtly hypothyroid patients and to investigate the possible effect of thyroxine replacement on insulin sensitivity, triglycerides and total cholesterol in those populations.

## MATERIALS AND METHODS

This study was approved by the Research and Ethics Committee of Asir Central Hospital and written informed consents were acquired from all participants.

Forty-two South Western Asian females were recruited from the endocrine clinic in a tertiary care hospital in southern region of Saudi Arabia, during January 2010 and December 2011. They included twenty seven patients with overt hypothyroidism and fifteen healthy euthyroid control women with matching age and body mass index

(BMI). Full history taking and clinical examination were done for all participants. The inclusion criteria were: adult, premenopausal females, who were newly diagnosed with overt hypothyroidism. Exclusion criteria were diabetes, polycystic ovarian disease, liver disorders, renal disorders, congestive cardiac failure or any other systemic illness. In addition, pregnancy and lactation, intake of oral contraceptive pills, statins and other medications that may alter thyroid functions, glucose homeostasis or serum lipids also accounted for exclusion from the study.

After an overnight fasting, blood samples were collected from all participants for measuring biochemical parameters. Thyroid profile (TSH, FT4 and FT3), fasting insulin, fasting plasma glucose, total cholesterol and triglycerides were measured, before and six months after initiating thyroxine therapy and reaching an euthyroid state for hypothyroid patients. These parameters were measured once for the euthyroid control group.

Insulin resistance (IR) was estimated using HOMA-IR,  $IR = FPG \text{ in milli-gram per deciliter} \times FI \text{ in micro-international unit per milli-litre}/405$ <sup>[15,16]</sup>. BMI was calculated by dividing weight of the patient in kilograms by square the height of the patient in meters<sup>[17]</sup>.

Thyroid profile and insulin level were estimated by Advia centaur auto-analyzer Siemens using chemiluminescent technology. Fasting plasma glucose and triglycerides were measured by bichromatic technique while cholesterol was measured by polychromatic technique. Normal ranges for all parameters: TSH: 0.27-4.2  $\mu$ IU/mL, FT4: 12-22 pmol/L, FT3: 3.9-6.8 pmol/L, FI: 2.6-37.6  $\mu$ IU/mL, total cholesterol: 50-200 mg/dL, triglycerides: 30-150 mg/dL<sup>[18-22]</sup>.

## Statistical analysis

Collected data were analyzed using the Statistical Package for Social Sciences (SPSS ver. 19). Descriptive statistics (*i.e.*, mean and standard deviation) were applied. Pearson's Correlation Coefficients ( $r$ ) between study variables were calculated. Significant *P*-values were considered at  $< 0.05$ .

## RESULTS

Our study population consisted of 42 females; 27 patients with overt hypothyroidism and 15 euthyroid healthy participants. The two groups had matching age and body mass index ( $33.12 \pm 10.4$  *vs*  $35.67 \pm 9.1$ ,  $P = 0.44$ ,  $31.11 \pm 6.78$  *vs*  $32.24 \pm 6.68$ ,  $P = 0.61$  respectively). Fasting insulin, FPG, IR, total cholesterol and triglycerides did not show significant difference in hypothyroid patients as compared to the euthyroid group ( $P$ -values 0.432, 0.621, 0.883, 0.586, 0.05 respectively) as shown in Table 1.

In the hypothyroid state, Triglycerides directly correlated to TSH and inversely to FT3 ( $P$ -value 0.009, 0.001 respectively). Total cholesterol inversely correlated to FT3 ( $P$ -value 0.029) and was directly proportionate to TSH although this relation did not reach statistical significance ( $P$ -value = 0.327) as shown in Table 2.

**Table 1 Laboratory and anthropometric parameters in hypothyroid patients versus euthyroid subjects**

Parameter	Hypothyroid (mean ± SD)	Euthyroid (mean ± SD)	P-value
Age (yr)	33.2 ± 10.4	35.7 ± 9.1	0.444
BMI	31.1 ± 6.8	32.2 ± 6.7	0.607
TSH	22.4 ± 36.2	2.9 ± 1.5	0.010
FT4	11.2 ± 4.0	13.7 ± 2.1	0.013
FT3	4.4 ± 1.0	4.5 ± 0.5	0.557
FPG	93.5 ± 14.7	89.8 ± 13.9	0.432
FI	10.6 ± 8.1	11.8 ± 6.3	0.621
IR	2.5 ± 2.1	2.6 ± 1.5	0.883
TG	144.8 ± 85.4	97.9 ± 36.1	0.050
TCH	195.0 ± 37.9	189.0 ± 29.9	0.586

BMI: Body mass index; TSH: Thyrotropin; FPG: Fasting plasma glucose; FI: Fasting insulin; IR: Insulin resistance; FT4: Free tetraiodothyronine; FT3: Free triiodothyronine; TG: Triglycerides; TCH: Total cholesterol.

**Table 2 Correlation between different variables before thyroxine replacement**

		TSH	FT4	FT3
TG	<i>r</i>	0.496	-0.321	-0.585
	<i>P</i>	0.009	0.102	0.001
TCH	<i>r</i>	0.196	-0.176	-0.420
	<i>P</i>	0.327	0.380	0.029

TSH: Thyrotropin; FT4: Free tetraiodothyronine; FT3: Free triiodothyronine; TG: Triglycerides; TCH: Total cholesterol; R: Relative coefficient.

After thyroxine replacement and attaining euthyroid state, there was no significant change in FPG or IR as compared to that before starting treatment (*P*-value = 0.216, 0.204 respectively) while FI significantly increased (*P* = 0.047). There was no significant change in triglycerides (*P*-value 0.175) meanwhile total cholesterol significantly decreased (*P*-value 0.043) as shown in Table 3.

## DISCUSSION

The association between hypothyroidism and diabetes mellitus had raised great interest in studying the mechanism of this association. Many studies targeted the influence of hypothyroidism on insulin sensitivity as the main underlying pathophysiology of this relation. Despite the many studies, results are conflicting with several studies reporting that hypothyroidism is a state of increased insulin resistance<sup>[11,23]</sup>.

In our study, there was no significant difference between the hypothyroid patients and the euthyroid healthy group regarding fasting insulin, FPG and insulin resistance. This is consistent with results of a study conducted by Giménez-Palop *et al*<sup>[24]</sup> on 17 hypothyroid women compared to 20 euthyroid control women.

Similarly, Owecki *et al*<sup>[25]</sup> did not find a significant difference in insulin sensitivity between hypothyroid patients and euthyroid participants.

Neither FPG nor insulin resistance as calculated by HOMA-IR significantly changed after thyroxine replace-

**Table 3 Comparison between different variables before and after thyroxine replacement**

Variable	Before treatment (mean ± SD)	After treatment (mean ± SD)	P-value
BMI	31.1 ± 6.8	31.4 ± 7.2	0.485
TSH	22.4 ± 36.2	3.0 ± 1.9	0.010
FT4	11.2 ± 4.0	14.5 ± 2.6	0.001
FT3	4.4 ± 1.0	4.7 ± 0.7	0.037
FPG	93.5 ± 14.7	90.2 ± 12.2	0.216
FI	10.6 ± 8.1	13.6 ± 7.3	0.047
IR	2.5 ± 2.1	3.0 ± 1.9	0.204
TG	144.8 ± 85.4	128.1 ± 64.8	0.175

BMI: Body mass index; TSH: Thyrotropin; FPG: Fasting plasma glucose; FI: Fasting insulin; IR: Insulin resistance; FT4: Free tetraiodothyronine; FT3: Free triiodothyronine; TG: Triglycerides; TCH: total cholesterol.

ment and reaching an euthyroid state as per clinical and laboratory evidence. There was a significant increase in the fasting insulin as compared to the pretreatment level but this was not statistically significant when compared to the euthyroid control ( $13.55 \pm 7.25$  vs  $11.82 \pm 6.31$ , *P* = 0.445) and it did not affect the overall calculated insulin resistance. This is again in agreement with results demonstrated by Giménez-Palop *et al*<sup>[26]</sup> although the increase in insulin levels in his study did not reach a statistical significance.

Referring to our study and studies in agreement with our findings, we can say that the association between hypothyroidism and T2DM may be attributed to a complex interplay<sup>[26]</sup>. It may depend on the severity of hypothyroidism<sup>[27]</sup>. There may be direct genetic links between thyroid diseases and T2DM as suggested by few studies. These studies suggest that homozygosity of polymorphism of the deiodinase type 2 (DIO2) gene, Thr92Ala is associated with an increased risk of T2DM<sup>[28]</sup>. Thyroid hormones may also affect glucose and lipid homeostasis *via* central effects at the level of the hypothalamus<sup>[29]</sup>.

Hypothyroidism is known to be associated with normal or high levels of triglycerides<sup>[30-32]</sup>. In our study, triglycerides in the hypothyroid patients did not differ significantly from the euthyroid control with direct proportion to TSH and inverse proportion to FT3<sup>[33]</sup>. Triglycerides did not significantly change after thyroxine replacement. This is in agreement with reports of several studies, which showed that triglycerides might be normalized or remain unchanged after treatment, suggesting a more complex cause of dyslipidaemia in hypothyroidism<sup>[34-43]</sup>.

Total cholesterol inversely correlated to FT3 in the hypothyroid patients with a significant decrease after thyroxine therapy. This is consistent with results obtained by Melpomeni *et al* who found that restoration of an euthyroid state in hypothyroid patients was associated with a significant reduction in total cholesterol<sup>[33,43]</sup>. Our findings are also consistent with those demonstrated in several other studies<sup>[35,37-41,44]</sup>.

The presence of some variations among different studies regarding the association between hypothyroidism and disturbed lipid profile may be explained by the variable effects of hypothyroidism on lipids according to the

severity of hypothyroidism in the studied groups of patients as evidenced by Sunanda *et al*<sup>[45]</sup>. Sunanda *et al*<sup>[45]</sup> studied the lipid profile in hypothyroid patients with different degrees of hypothyroidism and concluded that the effect of hypothyroidism on the serum lipids is more marked in patients with higher TSH levels.

So, the association between hypothyroidism and cardiovascular risk<sup>[45,46]</sup> may be attributed to the dyslipidaemic effect of hypothyroidism, underlying genetic factor or there may be another complex underlying mechanism that deserves further studies.

In conclusion, our study suggests that hypothyroidism has no impact on insulin sensitivity in overtly hypothyroid females of South Western Asian ethnicity. Thyroxine therapy does not cause significant change in insulin sensitivity in this ethnic group. So, other mechanisms that may explain the strong association between hypothyroidism and T2DM may exist. Although total cholesterol and triglycerides are not significantly higher in hypothyroid patients, thyroxine treatment leads to a significant reduction in total cholesterol without a significant effect on triglycerides. This may partially explain the association between hypothyroidism and cardiovascular risk.

## COMMENTS

### Background

Diabetes mellitus and hypothyroidism are the most common endocrine disorders. A strong association between both conditions exists. It was claimed that hypothyroidism increases the risk of developing diabetes mellitus through increased insulin resistance but studies in this field demonstrated conflicting data.

### Research frontiers

Recent evidences suggest that hypothyroidism is associated with dyslipidaemia and increased cardiovascular risk.

### Innovations and breakthroughs

The results presented herein show that in South Western Asian females, neither overt hypothyroidism nor thyroxine replacement has an effect on insulin resistance. Thyroxine therapy leads to a significant reduction in total cholesterol.

### Applications

The authors study indicates that the increased risk of diabetes mellitus in hypothyroid patients cannot be attributed to increased insulin resistance. So, investigating other mechanisms that may be involved is highly encouraged. Thyroxine therapy leads to a significant reduction in total cholesterol but it does not affect triglycerides. This partially explains the association between hypothyroidism and increased cardiovascular risk.

### Peer review

This is an interesting article about the effect of overt hypothyroidism and thyroxine therapy on insulin resistance and lipid profile in a specific ethnic population.

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# World Journal of *Diabetes*

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## Contents

Bimonthly Volume 4 Number 5 October 15, 2013

### EDITORIAL

- 162 Are diabetic patients being screened for sleep related breathing disorder?  
*Surani S*
- 165 Current status in diabetic macular edema treatments  
*Romero-Aroca P*
- 170 Chemo-profiling of eucalyptus and study of its hypoglycemic potential  
*Dey B, Mitra A*

### REVIEW

- 177 Diabetic cardiomyopathy: Pathophysiology, diagnostic evaluation and management  
*Pappachan JM, Varughese GI, Sriraman R, Arunagirinathan G*
- 190 Effects of glucagon-like peptide-1 receptor agonists on renal function  
*Filippatos TD, Elisaf MS*

### BRIEF ARTICLE

- 202 Effect of ethnicity on weight loss among adolescents 1 year after bariatric surgery  
*Messiah SE, Lopez-Mitnik G, Winegar D, Sherif B, Arheart KL, Reichard KW, Michalsky MP, Lipshultz SE, Miller TL, Livingstone AS, de la Cruz-Muñoz N*
- 210 Impaired fasting glucose: Pro-diabetic, "atheroprotective" and modified by metabolic syndrome  
*Onat A, Aydın M, Can G, Çakmak HA, Köroğlu B, Kaya A, Ademoğlu E*
- 219 High adiponectin levels fail to protect against the risk of hypertension and, in women, against coronary disease: involvement in autoimmunity?  
*Onat A, Aydın M, Can G, Köroğlu B, Karagöz A, Altay S*

### CASE REPORT

- 226 Sequential elevation of thyroglobulin and glutamic acid decarboxylase in type 1 diabetes  
*Kawasaki E, Yasui J, Tsurumaru M, Takashima H, Ikeoka T, Mori F, Akazawa S, Ueki, Kobayashi M, Kuwahara H, Abiru N, Yamasaki H, Kawakami A*

**APPENDIX** I-V Instructions to authors

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## Are diabetic patients being screened for sleep related breathing disorder?

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### Abstract

Prevalence of both diabetes mellitus and obstructive sleep apnea (OSA) is high among general population. Both of these conditions are associated with significant morbidity. OSA affects approximately 25% of men and 9% of women, and its prevalence is even higher among obese, Hispanics, African American and diabetic patients. Diabetes on the other hand besides having high prevalence in general population has even higher prevalence among ethnic populations as Hispanics and African American. Despite the availability of several simple screening tools for OSA, as Berlin questionnaire, STOP-BANG questionnaire, NAMES Criteria, the utility for screening of OSA among the diabetic population remains marginal. This in turn can lead to significant morbidity and complications related to OSA as well as worsening of diabetes mellitus and increase in diabetic complications due to untreated sleep related breathing disorder. It is therefore imperative for the primary care giver to screen for OSA among the diabetic population as a part of their routine evaluation to prevent worsening of diabetes, and its cardiovascular, renal, ophthalmologic and neurological complications.

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**Key words:** Obstructive sleep apnea and diabetes mel-

litus; Obstructive sleep apnea screening; Obstructive sleep apnea and metabolic syndrome

**Core tip:** There is higher prevalence of obstructive sleep apnea (OSA) among diabetic population; if undiagnosed and untreated can cause increase in diabetic complications. Primary care giver should routinely screen for OSA among diabetic patients as a part of their routine evaluations.

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### DIABETES

Obstructive sleep apnea (OSA) is characterized by repetitive, intermittent, complete or partial upper airway collapse, which leads to intermittent hypoxia, and sleep fragmentation. In addition, it also leads to increased hormonal fluctuations leading to an increased risk of hypertension, insulin resistance, heart attack, stroke and metabolic syndrome<sup>[1]</sup>. OSA is recognized as a chronic disorder affecting 24% of men and 9% of women in the general population, and its prevalence increases when looking at the older population<sup>[2]</sup>. Its prevalence is even higher among the obese population and diabetics reaches as high as 33%-77%<sup>[3]</sup>. Several screening questionnaires have been utilized for detection of sleep related breathing disorders. These include the Berlin Questions, NAMES Criteria, STOP-BANG questionnaires, American Society of Anesthesiologists (ASA), and the Sleep Disorder questionnaire. Sensitivities range from 83.6% for STOP-BANG to 86% in the Berlin questionnaire, and specificity from 38.2% for ASA to 77% with Berlin questionnaire<sup>[4-8]</sup>.

On the other hand, diabetes mellitus affects 6% of the American population and its incidence and prevalence is significantly worse among the both Hispanic and African-American population, suggesting an disproportionately increased burden of both diabetes and sleep disordered breathing/OSA in this ethnicity<sup>[9,10]</sup>. The number of individuals in the United States who have been diagnosed of diabetes mellitus approaches 24 million according to an estimate by the Center of Disease Control<sup>[11]</sup>. Several studies have shown an independent association between OSA and several components of metabolic syndrome, particularly in insulin resistance and abnormalities in lipid metabolism<sup>[12-15]</sup>.

Additionally, several screening devices with sensitivity and specificity ranging in the 90% have been in the market, such as ApneaLink, RUSleeping RTS, Embletta portable diagnostic system, and Stardust II<sup>[16-19]</sup>. Furthermore, several biomarkers and proteomics studies have been designed and are in the workup for the diagnosis of OSA<sup>[20]</sup>. Proteomic studies have used urine and serum based studies, utilizing 2 dimension gel based analysis of urine to check for uromodulin and urocortin-3. The serum based mass spectrometry assay is applied to check for different expressions of 103 proteins, which are expressed differently on the basis of severity of OSA<sup>[20-22]</sup>.

Several studies have shown a significantly high prevalence of OSA among patients with diabetes mellitus and metabolic syndromes, with prevalence ranging as high as 73% and 86%<sup>[5,23,24]</sup>. On the basis of these studies, approximately 17 million diabetic patients suffer from the metabolic syndrome in United States of America. In a study on prevalence of OSA and metabolic syndrome among the internal medicine setting, authors found the prevalence of OSA in patients with diabetes to be as high as 83%<sup>[25]</sup>. Moreover, in a study from Aronsohn *et al*<sup>[5]</sup> patients with OSA and diabetes mellitus had higher HBA1c levels when compared to diabetic patients without OSA. Several studies have shown an independent association between severity of OSA and insulin resistance<sup>[1,5,13,26]</sup>. Continuous positive airway pressure therapy on the other hand has shown improvement in insulin resistance in several studies<sup>[26]</sup>.

With the current obesity epidemics and the high prevalence of type 2 diabetes, it remains very surprising that screenings for OSA are not routinely done at primary care practitioner offices and diabetic clinics, despite the availability of several simple screening tools<sup>[6,8]</sup>. The data from the studies are clear regarding the adverse effects of sleep apnea on cardiovascular morbidity and mortality, as well as worsening of other diabetic complications in patients with untreated OSA. OSA and diabetes combinations have even more catastrophic cardiovascular, renal, and financial implications on health care if it remains undiagnosed and treated. The International Diabetes Federation recommends screening patients for possible OSA, assessing metabolic and cardiovascular risk factors and making appropriate referrals for the management in case of any abnormality detected<sup>[11]</sup>.

The author suggests an important role for the national, regional and local societies to educate the primary care practitioner and diabetes providers to make OSA screening a part of their armamentarium.

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## Current status in diabetic macular edema treatments

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**Core tip:** Diabetic macular edema is the leading causes of decreased visual acuity in diabetic patients, being the most important blindness causes in young adult people. New treatments have been developed in the last years, intravitreal anti-vascular endothelial growth factor drugs, corticoid intravitreal implants or injections, but the laser photocoagulation being the gold standard of diabetic macular edema treatment. The following manuscript tries to clarify the current status of diabetic macular edema treatment.

### Abstract

Diabetes is a serious chronic condition, which increase the risk of cardiovascular diseases, kidney failure and nerve damage leading to amputation. Furthermore the ocular complications include diabetic macular edema, is the leading cause of blindness among adults in the industrialized countries. Today, blindness from diabetic macular edema is largely preventable with timely detection and appropriate interventional therapy. The treatment should include an optimized control of glycaemia, arterial tension, lipids and renal status. The photocoagulation laser is currently restricted to focal macular edema in some countries, but due the high cost of intravitreal drugs, the use of laser treatment for focal and diffuse diabetic macular edema (DME), can be valid as gold standard in many countries. The intravitreal anti vascular endothelial growth factor drugs (ranibizumab and bevacizumab), are indicated in the treatment of all types of DME, but the correct protocol for administration should be defined for the different Retina Scientific Societies. The corticosteroids for diffuse DME, has a place in pseudophakic patients, but its complications restricted the use of these drugs for some patients. Finally the intravitreal interface plays an important role and its exploration is mandatory in all DME patients.

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### INTRODUCTION

There has been a dramatic increase in the incidence of diabetes mellitus (DM) worldwide, which has been exacerbated by the growing obesity problem across the globe. The World Health Organization (WHO, estimated 30 million people worldwide had some form of diabetes in 1985; by 2000, the number had increased to 177 million. The WHO projections suggest that the number of people suffering from the disease will increase to some 370 million by 2030<sup>[1]</sup>.

Diabetes is a serious chronic condition, which increase the risk of cardiovascular diseases, kidney failure and nerve damage leading to amputation. Furthermore the ocular complications include diabetic retinopathy is a microvascular complication of diabetes that primarily affects capillaries, which is the leading cause of blindness among adults in the industrialized countries, affecting from 2% to 5% of the entire population<sup>[2,3]</sup>. The causes

of visual decrease include proliferative diabetic retinopathy and diabetic maculopathy; the last condition include ischemia of the macula due to retinal capillary occlusion around the macula, increasing the foveal avascular zone in fluorescein angiography, but the most frequent causes of visual acuity decrease in diabetes is due to diabetic macular edema (DME), which is the leading cause of blindness in young adults in developed countries, affecting 12% of type 1 and 28% of type 2 diabetic patients<sup>[4]</sup>. Attending the increase of DME at 2030, the DME prevalence can increased to 100 million of patients. Despite of diabetic macular edema can have a spontaneous recovery (it is important to recognize that about 33% to 35% of patients resolve DME spontaneously after six months without treatment<sup>[3,5]</sup>), the treatment of patients who developed DME, has become the most important focus in the DM patient's treatment,

The medical DME treatment should be based on a good control of glycemia, arterial hypertension, lipids (it is important the role of low-density lipoprotein (LDL)-cholesterol in DME development, particularly in exudates macular deposits), and renal function<sup>[6,7]</sup>. However, despite the systemic metabolic control values being essential for patients with diabetic retinopathy, it has proven to be insufficient for DME if it appears, and we have to take additional measures, in order to avoid the subsequent loss of vision. While laser treatment of DME has been a valid treatment so far, in some cases it has been inadequate. The introduction of new treatments, such as intravitreal corticosteroids or anti-vascular endothelial growth factor (VEGF) drugs have recently shown their safety and efficacy, and together with laser photocoagulation are becoming the treatments of choice in the management of DME.

## CONCEPT OF FOCAL VERSUS DIFFUSE DIABETIC MACULAR EDEMA

DME is further classified into focal or diffuse, depending on the leakage pattern seen on the fluorescein angiogram (FA). In focal DME, discrete points of retinal hyperfluorescence (leakage of intravascular liquid to interstitial space, due a vasopermeability) are present on the FA due to focal leakage of microaneurysms, which are the cause of retinal thickening. Commonly, these microaneurysms are surrounded by circular hard exudates. A variation of this form is the multifocal macular edema, which in some cases is confused with diffuse macular edema. This form appears under fluorescein angiography as multiple foci of leakage due to the presence of multiple foci of microaneurysms.

In diffuse DME, there are areas of diffuse leakage on the FA due to intraretinal leakage from dilated retinal capillary bed and/or intraretinal microvascular abnormalities (IRMA), and/or from arterioles and venules without foci of leaking microaneurysms<sup>[8]</sup>. To treat DME, it is important to use the classification by Bresnick *et al*<sup>[9]</sup> into focal or diffuse DME, modified by Browning *et al*<sup>[10]</sup>.

## LASER TREATMENT

Laser treatment was defined by the ETDRS study in its Reports number 3 and number 4; there are two different techniques<sup>[5,11]</sup>: (1) Focal laser. Focal treatment is required for focal lesions located between 500 and 3000  $\mu\text{m}$  from the centre of the macula. The term "focal lesions" according to the ETDRS classification includes: microaneurysms, IRMA and short capillary segments that show focal fluorescein leakage. The treatment consists of burns of 50-100  $\mu\text{m}$  of moderate intensity and 0.05-0.1 s duration, the end point of treatment is whitening or darkening of focal lesions. Microaneurysms below 40  $\mu\text{m}$  in diameter had successful results with low laser intensity, but microaneurysms with more than 40  $\mu\text{m}$  diameter need more intense laser burns (a more whitening result) and sometimes need a re-treatment. The clusters of microaneurysms, in particular those with in hard exudate rings, may be treated with larger spots (200-500  $\mu\text{m}$ ), with subsequent re-treatment of any large microaneurysms within the cluster with 50  $\mu\text{m}$  spots to obtain darkening or whitening. The treatment of lesions of more than 3000  $\mu\text{m}$  from the centre is recommended if prominent leaks are present and associated with retinal thickening or hard exudates that extend closer to the centre; and (2) Grid laser, in which mild power laser impacts were made with a spot size of 50-200  $\mu\text{m}$ , for a duration of 0.05-0.5 s obtained a mild retinal pigment epithelium whitening, with power adjusted to prevent the burns from spreading to more than 200  $\mu\text{m}$  in diameter. Grid treatment is not placed within 500  $\mu\text{m}$  of the centre of the macula or within 500  $\mu\text{m}$  of the disc margin, but may be placed in the papillomacular bundle. Grid can extend up to 2 disk diameters (3000  $\mu\text{m}$ ) from the centre of the macula or to border panretinal photocoagulation treatment, if present. Any focal leaks within the areas of the grid treatment are treated focally. The burns are placed approximately two visible burn widths apart in the areas of the macular edema (retinal thickening) that are thought to be related to diffuse leakage or capillary loss.

Laser photocoagulation is not a harmless technique, and side effects appear secondary to the burn induced in the retinal layers, in particular, the destruction of the retinal pigment epithelium might induce apoptosis of the surrounding retinal cells. In the macular area, some secondary effects might affect the visual acuity. One the most important effects that can decrease visual acuity is the enlargement of a laser scar, referred to as "atrophic creep", which might threaten the visual prognosis if the laser is applied too close to the fovea Schatz *et al*<sup>[12]</sup> reported that enlarged laser scars reached the central fovea in 11 of 203 eyes with diabetic macular edema after grid laser photocoagulation. Brancato *et al*<sup>[13]</sup> reported that the scars enlarged by an average of 103% after treatment of choroidal neovascularization in degenerative myopia. The Maeshima *et al*<sup>[14]</sup> study showed that the expansion rate of laser scarring was higher in the posterior pole (12.7%) than in the midperiphery (7.0%). The authors explain that because the density of the photoreceptors

is higher in the posterior pole, more photoreceptors are destroyed in the posterior pole than in the midperiphery when using the same spot size of laser photocoagulation. Furthermore, the photoreceptors interact with surrounding photoreceptors through horizontal or amacrine cells; thus, the authors hypothesized that necrosis of regional photoreceptors may lead to apoptosis of surrounding cells, which might explain why laser scars gradually expand at a higher rate in the posterior pole.

At present, despite the enthusiasm for evaluating several new treatments for DME including intravitreal therapies for DME (*e.g.*, corticosteroids, and anti-VEGF drugs), laser photocoagulation remains the gold standard of care and the only treatment with proven efficacy in a large-scale clinical trial for this condition. We can consider a best practice, use the focal laser photocoagulation for focal DME as the first choice treatment, and also in many countries, we can consider the use of grid laser for diffuse macular edema treatment.

## INTRAVITREAL ANTI-VEGF DRUGS

The vascular endothelial growth factor belongs to a family of different growth factors (types: A, B, C and D). The type VEGF-A are present in ocular inflammatory pathologies, and it has become accepted as one of the most potent factors in the induction of angiogenesis. Six major isoforms of VEGF-A exist: 121, 145, 165, 183, 189 and 206. VEGF-A 165 is the most important factor in the pathophysiology of DME<sup>[15,16]</sup>. The VEGF is produced by endothelial cells, pericytes, and neuronal cells as: ganglion cells, Müller cells, and glial cells. The upregulation of VEGF is produced by hypoxia, hyperglycemia (which itself can enhance the response of retinal cells) and cytokines, such as insulin-like growth factor 1, interleukin-6, and protein kinase C-beta. The VEGF induce angiogenesis, vasculogenesis, inflammation, chemotaxis and increase of vascular permeability developing disruption of hemato-retinal barrier, and subsequent DME<sup>[17,18]</sup>. The use of anti-VEGF drugs in DME treatment has been corroborated by many studies, which demonstrate the validity of its use in DME<sup>[19-22]</sup>. In many countries the use of anti VEGF drugs (bevacizumab or ranibizumab) has been extended, and it's common practice as first choice in some patients. Despite the most important limitation of its use, is the high cost of intravitreal drugs<sup>[23]</sup>.

## INTRAVITREAL STEROID INJECTION

### *Role of the inflammation in DME*

Inflammation is a nonspecific response to injury that includes a variety of functional and molecular mediators, including recruitment and activation of leukocytes. Many of the molecular and functional changes that are characteristic of inflammation have been detected in retinas from diabetic patients. The DME increases expression of intercellular adhesion molecule 1 in the retina, and produces an interaction between this adhesion molecule on

retinal endothelia with the CD 18 adhesion molecule on monocytes and neutrophils, contributing to the diabetes-induced increase in leukostasis within retinal vessels. This attraction and adhesion of leukocytes to the vascular wall are important components of inflammatory processes. Furthermore, leukostasis can contribute to the development of capillary nonperfusion in retinal vessels, and it has been postulated that leukostasis is a factor in the death of retinal endothelial cells<sup>[18]</sup>.

### *Place of intravitreal corticosteroids in DME treatment*

The use of corticosteroids as a means to treat ocular DME has emerged as an increasingly common treatment for certain patients. The Diabetic retinopathy clinical research network (DRCRnet), reported 2-years' results of a multicentered, randomized, clinical trial comparing preservative free intravitreal triamcinolone (TA) and focal/grid laser for DME<sup>[24]</sup>. This randomized study indicates clearly that focal/grid laser is a better treatment than intravitreal TA in eyes with DME with VA between 20/40 and 20/30. The most frequent intravitreal TA complication is an increase in intraocular pressure (observed in 30% of patients) and cataract formation. From this study and other non-randomized studies, we suggest that intravitreal TA is a promising therapy method for DME that is unresponsive to laser photocoagulation and for patients previously submitted to cataract surgery.

Another corticosteroid currently used but without DME treatment indication for any international agency, is the dexamethasone intravitreal implant<sup>[25]</sup>. The most important difficulty in this type of study is the safety and drug release profiles of this injectable implant. Further studies are warranted to assess its long-term efficacy and safety.

## TREATMENT OF REFRACTORY DME

As we said previously the DME is a chronic disease, that becomes difficult to treat in some patients, the refractory DME has become one of the biggest problems for the retina specialist. First we take into account the importance of systemic diabetes status control, we should control strictly the glycaemia, arterial tension, lipids and renal status<sup>[26]</sup>, furthermore we should revise the diabetes treatment, because some drugs can induce DME as glitazone<sup>[27,28]</sup>. Despite of some patients seems that all is correct, in these cases which has all metabolic parameters correct and which has been treated by other therapies but the DME not decreased, is important to examine the vitreo-retinal surface.

### *Importance of vitreo-retinal interface*

Clinical evidence indicates that the vitreo-retinal interface may play a role in the pathogenesis of DME, the persistent vitreo-macular traction by vitreous cortex before posterior vitreous detachment (PVD), or the persistence of residual cortical vitreous (vitreoschisis) after PVD, and thickened and taut posterior hyaloid that may be adherent

to internal limiting membrane (ILM), with a subsequent macular traction. In the macular area the vitreous and ILM have the strongest attachment and the ILM (which is the basement membrane of the Müller cells) is thinnest. A densely-packed collagen filament of posterior vitreous cortex penetrates the ILM in the macular area. Vitrectomy, removing all the posterior vitreous cortex and ILM peeling has been shown to improve visual acuity and decrease macular thickening<sup>[29]</sup>.

### Vitreous surgery

Laser photocoagulation has no place in cases of tractional or taut DME. In such cases there is clinical evidence that vitrectomy will resolve the DME. The beneficial mechanisms may be: to remove AGE ligand-induced mechanical traction between the posterior cortical vitreous and the ILM of macula and to remove AGE that may also inhibit the activation of the RAGE axis and its pro-inflammatory effects.

Currently the discussion is centered on ILM peeling and its usefulness. It is not clear that ILM peeling is necessary for tractional-DME treatment as it may hinder the formation of epiretinal membranes but may help to remove all the cortical vitreous that may otherwise be left behind even after the posterior hyaloid is removed<sup>[30]</sup>. The complications encountered after vitrectomy include cataract, retinal detachment, epiretinal membrane, glaucoma, and vitreous hemorrhage.

## CONCLUSION

Today, blindness from diabetic macular edema is largely preventable with timely detection and appropriate interventional therapy. The treatment should include an optimized control of glycemia, arterial tension, lipids and renal status.

The photocoagulation laser is currently restricted to focal macular edema in some countries, but due the high cost of intravitreal drugs, the use of laser treatment for focal and diffuse DME, can be valid as gold standard in many countries. The intravitreal anti VEGF drugs (ranibizumab and bevacizumab), are indicated in the treatment of all types of DME, but the correct protocol for administration should be defined for the different Retina Scientific Societies. The corticosteroids for diffuse DME, has a place in pseudophakic patients, but its complications restricted the use of these drugs for some patients. Finally the intravitreal interface plays an important role and its exploration is mandatory in all DME patients.

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## Chemo-profiling of eucalyptus and study of its hypoglycemic potential

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### Abstract

Constant escalations in the number of diabetics worldwide and the failure of conventional therapy to restore normoglycemia without adverse effects, in spite of tremendous strides in modern medicine, calls for naturopathy and alternative medicine. Because diabetes is multi-factorial and has secondary complications, prevention of hyperglycemia is the central dogma for its management. To date, no oral hypoglycemic exists which can achieve tight glycemic control without side effects. Dietary adjuncts, lifestyle interventions and a resurgence of interest in phyto-therapy have consequently gained ground. Natural hypoglycemics have attracted attention due to ease of incorporation in everyday diet, affordability, less adverse effects, and long term safety. Ethno botanical literature reports more than 800 anti-diabetic plants species. Eucalyptus is well represented in the Aboriginal Pharmacopoeias for its various pharmacological activities. Its hot aqueous decoction has been used as a hypoglycemic in various regions of world. This editorial attempts to summarize the data on the hypoglycemic potential of the different eucalyptus species, highlight the value of its natural biomolecules for the prophylaxis and treatment of type 2 diabetes, describe their mechanistic actions, shed light on the posology and safety aspects of eucalyptus

and assess its applicability as a reinforcement to currently used therapy.

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**Key words:** Normoglycemia; Escalations; Eucalyptus; Central dogma; Dietary adjuncts; Aboriginal Pharmacopoeias; Natural biomolecules; Prophylaxis; Posology

**Core tip:** Eucalyptuses, indigenous to various countries of the world has been described in various Aboriginal, British and European Pharmacopoeias with its wide range of phytochemicals demonstrating a range of pharmacological effects including hypoglycemic action. The volatile and non-volatile constituents of Eucalyptus include terpenes and tri-terpenoids, flavonoids, flavanols, gallotannins, quercetin, euglobals, procyanidins, macro carpals. Pharmacopoeias have reported uses of hot eucalyptus leaf decoctions as tea to act as hypoglycemic agents. This editorial attempts to assess and highlight eucalyptus species with anti-diabetic potential and the probable biomolecules contributing to this along with their mechanistic role, as well as their posology and safety.

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### INTRODUCTION

The world diabetic population is expected to show a steady growth of 366 million by 2030, despite the availability of insulin therapy and several synthetic hypoglycemics. Also, the severe side effects associated with the current treatment options cannot be neglected on a long-term basis<sup>[1,2]</sup>. Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia with

**Table 1** Contrasting clinical and pathophysiologic features of types 1 and 2 diabetes

Features	Type 1 DM	Type 2 DM
Age at onset	Early, below 35 yr	Late, after 40-45 yr
Type of onset	Abrupt and severe	Gradual and insidious
Frequency of occurrence	10%-20%	80%-90%
Family history	Less than 20%	About 60%
Pathogenesis	Autoimmune destruction of $\beta$ -cells	Insulin resistance, impaired insulin secretion
Body weight	Normal	Obese/non-obese
Genetic locus	Unknown	Chromosome 6
Condition of islet cells	Insulinitis, $\beta$ -cell destruction	No insulinitis, later fibrosis of islets
Blood insulin level	Decreased insulin	Normal or increased insulin
Clinical management	Insulin and diet	Insulin, oral drugs, diet, exercise

DM: Diabetes mellitus.

disturbance of carbohydrate, protein or fat metabolism resulting from defects in insulin secretion, insulin action or both (World Health Organization, WHO, 1999). DM and the major complications associated with it such as retinopathy leading to blindness, diabetic foot ulcers necessitating limb amputations, neuropathy, nephropathy leading to end stage renal disease, is becoming the third greatest threat to the health of mankind after cancer, cerebrovascular and cardiovascular diseases. DM not only takes a heavy toll of lives around the world but imposes a serious financial burden on the sufferers and their family members. There are two main types of DM having contrasting clinical and pathophysiologic features; type 1 or insulin dependent diabetes mellitus (IDDM), and Type 2 or non-IDDM both (Table 1). Type 2 diabetes is found to be more prevalent, occurring mostly due to a combination of insulin resistance and inadequate compensatory insulin secretory response<sup>[3-6]</sup>.

Pathophysiologically, type 2 DM, unlike type 1 DM, does not involve autoimmune destruction of pancreatic  $\beta$ -cells but involves multiple disturbances in glucose homeostasis including impaired insulin secretion, peripheral insulin resistance mostly in muscles, liver and adipocytes, and abnormalities in liver glucose uptake. The pancreas of type 2 diabetics produces insulin, yet insulin resistance prevents its proper use at the cellular level. Glucose cannot enter target cells and accumulates in the bloodstream, resulting in hyperglycemia. The high blood glucose levels often stimulate an increase in insulin production by the pancreas; thus, type 2 diabetic individuals often have excessive insulin production or hyperinsulinemia. Insulin is a potent anti-lipolytic hormone and restrains the release of free fatty acid (FFA) from the adipocytes by inhibiting the enzyme hormone sensitive lipase<sup>[6]</sup>. The fat cells of type 2 diabetics are markedly resistant to the inhibitory effect of insulin on lipolysis and, despite 2-4 fold increments in plasma insulin levels, the rate of lipolysis in post absorptive phase is still high. The availability of exogenous insulin to inhibit the elevated

basal rate of lipolysis and to reduce the plasma FFA concentration is also markedly impaired. The pathogenicity is shown to be further aggravated by the circulating triglycerides which have been shown to impair insulin action in both liver and muscle. The glucose transport mechanism is severely impaired in the adipocytes and muscles of type 2 diabetics. Glucose transporter subtype 4 (GLUT4), mRNA and protein content are markedly reduced and the ability of insulin to elicit a normal translocation response and to activate the GLUT4 transporter after insertion into the cell membrane is decreased<sup>[3-6]</sup>.

Although several synthetic hypoglycemics have been developed a safe and effective treatment paradigm is yet to be developed. Herbs are rich sources of bio-active compounds with versatile pharmacology and WHO has recommended traditional plant treatment for diabetes because of their safety, effectivity, availability and affordability. Moreover herbs can be used as dietary adjuvants. Hence there has been a great resurgence of interest in phyto-therapy with the NAPRALERT database (NATURAL PRODUCTS ALERT) and the ethno-botanical literature reporting more than 800 anti-diabetic plant species<sup>[3-5,7,8]</sup>. Amongst these, this paper attempts to highlight the hypoglycemic potential of the different species of Eucalyptus. This is a diverse genus of flowering trees and shrubs, taxonomically from family Myrtaceae, indigenous to Australia, Tasmania and cultivated mostly in sub-tropical and warm temperate regions of the world. From ancient times the bark and leaves of different species of eucalyptus have been used as folk medicine for the treatment ailments such as cold, fever, toothache, diarrhea and snake bites. Popularly known as “gum tree”, the use of eucalyptus as “herbal tea” has been recorded in Aboriginal, European and British Pharmacopoeias. Aqueous hot eucalyptus leaf decoctions have been used as a traditional remedy for DM<sup>[9]</sup>. Though there are over 500 species of eucalyptus this editorial will focus on four species viz. *Eucalyptus globulus* (*E. globules*) (Blue gum or Tasmanian blue gum), *Eucalyptus citriodora* (*E. citriodora*) (Lemon scented gum), *Eucalyptus camaldulensis* (*E. camaldulensis*) (River red gum or Murray red gum) and *Eucalyptus tereticornis* (*E. tereticornis*) (Forest red gum) with the chemo-profiling of their phyto-constituents, study of their mechanistic role as hypoglycemic agents and discussion of their status in alternative anti-diabetic therapy<sup>[9,10]</sup>.

## PHYTO-CHEMICAL PROFILING OF EUCALYPTUS

Eucalyptus contains both volatile and non-volatile fractions; amongst which the terpenoids are one of the major components comprising most of the essential oil of eucalyptus (eucalyptus oil), imparting its characteristic odor. The volatile, essential oils obtained by steam distillation comprise no less than 70% of 1,8-cineole, as reported by the pharmacopoeias of Britain, France, United States, China *etc.*. Other volatile oil constituents include  $\alpha$ -pinene,

**Table 2** Phyto-chemicals and pharmacology of four eucalyptus species

Eucalyptus species	Major phyto-chemicals	Pharmacological actions
<i>E. globulus</i> (blue gum/tasmanian blue gum)	Euglobals, essential oils (1, 8 cineole, carvone, citral, citronellal, geranyl acetate, $\alpha$ -pinene, $\alpha$ -pinocarvone, $\beta$ -pinene), hydrocarbons (4-hydroxytritiacontane-16, 18-dione, 16-hydroxy B tritiacontanone, n-Tritiacontane 16, 18-dione), Macrocarpals H, I, J	Anti-diabetic, anti-bacterial, anti-plaque, anti-tumor, anti-viral, anti-fungal, anti-histaminic, anti-inflammatory, anti-oxidant, anti-malarial
<i>E. citriodora</i> (temon scented gum)	Essential oils (cineole, citronellal, citronellic acid), sterols ( $\beta$ 9-sitosterol)	Anti-diabetic, analgesic, anti-fungal, anti-inflammatory, bone resorption inhibition, natural repellent
<i>E. camaldulensis</i> (river red gum/murray red gum)	Essential oil (aromadendrene, myrtenal, borneol, camphene, carvacrol, citronellal, citronellyl acetate, cryptone- $\alpha$ , terpenyl acetate), Flavonoids (apigenin, chrysin, flavone, luteolin, eriodictyol, hesperetin, naringenin, pinoem-brin), triterpenoids (oleanolic acid, maslinic acid, camaldulic acid, camaldulenic acid)	Anti-diabetic, anti-microbial, anti-nociceptive, anti-oxidative, cytotoxic
<i>E. tereticornis</i> (forest red gum)	Essential oils (1, 8-cineole, camphene, carvone, citral, citronellal, geranyl acetate, limonene, linalool oxide), phloroglucinol monoterpene derivatives (euglobal-T1, euglobal II c), urosolic acid, triterpene esters (tereticornate A and B)	Anti-diabetic, hepatoprotective, myorelaxant

*E. globules*: *Eucalyptus globules*; *E. citriodora*: *Eucalyptus citriodora*; *E. camaldulensis*: *Eucalyptus camaldulensis*; *E. tereticornis*: *Eucalyptus tereticornis*.

p-cymene,  $\gamma$ -terpinene. Terpenes like bicyclogermacrene,  $\beta$ -phellandrene are also reported. The chief constituents of the essential oil of *E. globulus* include: 1,8-cineole/eucalyptol (above 72%),  $\alpha$ -terpineol, terpinen-4-ol, linalool,  $\alpha$ -pinene,  $\beta$ -pinene, globulol, epiglobulol<sup>[10-12]</sup>. Citronellal, is the major component of the essential oil of *E. citriodora*, the other components being cis-geraniol, citronellol acetate,  $\beta$ -bisabolene, Dihydrocarveol acetate, 3-hexen-1-ol, Pregn-5-en-20-one, 3,17-dihydroxy-3-acetate. The major components of the essential oil of *E. tereticornis* are: eucalyptol, 1R- $\alpha$ -pinene, isopinocarveol. The essential oil of *E. camaldulensis*: dihydrocarveol acetate, (-)-spat-hunelol, cis-nerolidol, megastigma-3, 7 (Z), 9-triene, thymol, aromadendrene,  $\alpha$ -pinene,  $\alpha$ -terpineol, drimenol, cubenol. Bioactives isolated from *E. camaldulensis* include pentacyclic triperpenoid, camaldulin along with ursolic acid lactone acetate and ursolic acid lactone; eucalyptanoic acid a triterpenoid acid; several flavonoid glycosides<sup>[10-14]</sup>.

Non-volatile constituents like flavonoids, triterpenoids, and tannins have been isolated from various eucalyptus species. An important group of phenolic compounds of eucalyptus are the formylated phloroglucinols, notable amongst which are euglobals, macrocarpals, sideroxytonals, robustadials. These show a wide range of biological actions including anti-bacterial, antioxidant, anti-inflammatory, HIV-RTase inhibitory, anti-malarial and anti-tumor promoting activities<sup>[9-11,15,16]</sup>. Osawa and Namiki<sup>[17]</sup> reported the presence of strong antioxidants in the leaf wax of a range of eucalyptus species. 4-hydroxytritiacontane-16, 18 dione, a very powerful antioxidant, was isolated from the leaf wax of *E. globulus* by Osawa and Namiki<sup>[18]</sup> and makes up more than 0.3% of the leaf wax content. *E. globulus* could serve as an abundant source of powerful antioxidants. Phenolics including tannins, flavonol glycosides, acylated flavonol glycosides have been isolated from the species *E. camaldulensis*. Five bioactive compounds, macrocarpals A-E, detected in the ethanol extracts of the leaves of *E. globulus* showed HIV-RTase inhibitory activity<sup>[9-11]</sup>. Along with their antibacterial actions, macrocarpals A, B, C, D in the

concentration range of 2-2.8  $\mu$ mol have been shown to inhibit aldose reductase, the target enzyme in the control of diabetic complications<sup>[9,10,18-21]</sup>. Euglobals are cyclo-adducts of formyl phloroglucinol. Euglobal-III was first isolated from buds of *E. globulus* and the presence of Euglobal-T1 has been reported in *E. tereticornis*. Twelve euglobals isolated from the leaves of *E. globulus* showed stronger anti-inflammatory potential than indomethacin and similar inhibitory effects to berberine. Flavonols (quercetin, myricetin, kaempferol), proanthocyanidins, anthocyanins have been isolated from leaves of the eucalyptus species<sup>[9-13,15,16,18-20]</sup>.

Cumulative details of the active phyto-constituents of the four eucalyptus species are provided in Table 2.

One important therapeutic intervention in the treatment of diabetes the reduction of post-prandial hyperglycemia by inhibiting the actions of carbohydrate hydrolyzing enzymes like  $\alpha$ -amylase and  $\alpha$ -glucosidase. Several biomolecules of the phyto-kingdom have been found to be potent  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors<sup>[22-25]</sup>. Amongst the major components isolated from the eucalyptus species under study the flavonoids, (mostly quercetin, kaempferol, myricetin), phenolics (including tannins, ellagic acid, gallic acid), and terpenoids (including ursolic acid, oleanolic acid, p-cymene, 1, 8-cineole, 1-(S)- $\alpha$ -pinene) are found to exhibit strong  $\alpha$ -amylase activity while polyphenols, proanthocyanidins, anthocyanins are found to be potent natural  $\alpha$ -glucosidase inhibitors. Other enzymes such as dipeptidyl peptidase 4 (DPP4), aldose reductase (AR), angiotensin converting enzyme (ACE), and peroxisome proliferator activated receptor (PPAR)- $\gamma$  also play significant roles in diabetes<sup>[16]</sup>. AR, a member of the aldo-keto-reductase super family, is the first and rate limiting enzyme in the polyol pathway and reduces glucose to sorbitol, utilizing NADPH as a cofactor. Sorbitol is then metabolized to fructose by sorbitol dehydrogenase. In DM, due to increased availability of glucose in insulin in-sensitive tissues such as lens, nerves, retina there is increased formation of sorbitol through the polyol pathway. Intracellular accumulation of sor-

bitol is implicated in chronic complications of diabetes including cataract, retinopathy and neuropathy. AR-inhibitors prevent the conversion of glucose to sorbitol and are able to control diabetic complications<sup>[16]</sup>. Limited literature data have shown that natural biomolecules with potent aldose reductase inhibitory actions are: flavonoids like Quercetin, Myricitrin, coumarins, monoterpenes, stilbenes *etc.*<sup>[16,26]</sup>. Molecular docking analyses have shown that the binding energies of phyto-chemicals like myrcene, citral, geraniol (-8.76, -7.24 and -7.93 kcal/mol respectively) are sufficient to inhibit the activity of aldose reductase<sup>[27]</sup>. Results of *in-silico* docking studies have shown that flavonoids with binding energy ranging between -9.33 kcal/mol to -7.23 kcal/mol contributed to AR inhibitory properties<sup>[28]</sup>. The four species of eucalyptus chosen for study of hypoglycemic potential to contain a number of flavonoids and monoterpenes and AR inhibition may be one of the possible modes for the hypoglycemic actions of eucalyptus<sup>[16,26]</sup>.

Increase in the level of reactive oxygen species (ROS) is another pathogenic factor in type 2 diabetes. Attenuation in ROS level may be due to increased production/diminished depletion by enzymic catalase, glutathione peroxidase, and superoxide dismutase antioxidants. Natural antioxidants which scavenge free radicals may be acting synergistically with their hypoglycemic activity in exerting an overall anti-diabetic action. The presence of powerful antioxidant compounds in *E. globulus* contributes significantly to its hypoglycemic potential<sup>[16,18-20]</sup>.

PPAR- $\gamma$  is a key receptor in lipid and glucose homeostasis because of its ability to reduce the plasma FFA. Phyto molecules can exert their insulin sensitizing actions through their high affinity for the receptor PPAR- $\gamma$  and hence can act as therapeutic targets for type 2 diabetes. Terpenoids are found to act as PPAR modulators regulating carbohydrate and lipid metabolism and are hence a promising therapeutic target for type 2 diabetes. Chemo-profiling of the eucalyptus species under study depicted the presence of several terpenoids and PPAR antagonism may also be a possible mode for the hypoglycemic action of eucalyptus<sup>[29]</sup>. ACE is an important enzyme involved in vascular tension and is hence associated with hypertension, a long term complication of diabetes. Along with oxidative stress, ACE plays a key role in diabetes. ACE activates histidyl leucine dipeptide (Angiotensin- I) into the potent vasoconstrictor Angiotensin- II. Angiotensin- II influences the release of aldosterone, which increases blood pressure by promoting sodium retention in distal tubules. Thus, biomolecules with ACE inhibitory activities can be considered as useful therapeutic targets against diabetes, as evidenced by United Kingdom Prospective Diabetes Study study<sup>[30]</sup>. Natural biomolecules like flavonoids, flavonols, anthocyanins, tri-terpenes are found to be potent ACE inhibitors, and find use in controlling hypertension, one of the problems associated with DM. Molecular docking studies also indicate the use of herbal ACE inhibitors in the management of DM<sup>[31]</sup>.

Glucagon-like peptide-1 (GLP-1) is a remarkable anti-diabetic gut hormone with its combined actions of stimulating insulin secretion, increasing beta cell mass, inhibiting glucagon secretion, reducing the rate of gastric emptying and inducing satiety. GLP-1 is rapidly deactivated by DPP4 and animal studies have shown that inhibition of DPP4 improves glucose tolerance and increases insulin secretion. Thus, natural biomolecules with DPP4 inhibitory activity will help to increase the levels of endogenous GLP-1 activity and act as an important therapeutic bullet against type 2 diabetes. Molecular docking studies have suggested the use of herbal DPP4-inhibitors as a therapeutic target against diabetes and suggest that tri-terpenoids, steroids and phenolic constituents are mainly responsible for the activity<sup>[32]</sup>.

## HYPOGLYCEMIC POTENTIAL OF EUCA-LYPTUS

Although the eucalyptus species exhibits various pharmacological actions (Table 2) the central focus of this editorial is on the hypoglycemic potential of eucalyptus. *E. globulus* is used in the traditional treatment of diabetes. In STZ-induced diabetic mice, incorporation of *E. globulus* in diet (62.5 g/kg) and drinking water (2.5 g/L) reduced the hyperglycemia and associated weight loss. Gray *et al.*<sup>[33]</sup>, suggested that an aqueous extract of *E. globulus* (0.5 g/L) enhanced 2-deoxy-glucose transport by 50%, glucose oxidation by 60%, and incorporation of glucose into glycogen by 90% in abdominal muscle of mice and a 20 min incubation of the same extract (0.25-0.5 g/L) evoked a step-wise, 70%-160% enhancement of insulin secretion from the clonal pancreatic beta-cell line. In 2009 Basak *et al.*<sup>[34]</sup>, studied the anti-diabetic actions of the essential oil of *E. camaldulensis* and found that it inhibited both  $\alpha$ -amylase and  $\alpha$ -glucosidase in a non-competitive manner and also exhibited greater antioxidant potential than butylated hydroxyl toluene and curcumin, giving further evidence in support of its hypoglycemic actions. Nakhac *et al.*<sup>[35]</sup> evaluated the effects of *E. globulus* (20 g/kg in diet and 2.5 g/L in drinking water) on lipid peroxidation, protein oxidation and antioxidant power in plasma and liver homogenate as well as glycated hemoglobin. They suggested that *E. globulus* possess antidiabetic and antioxidant property, reduces oxidative stress mostly by reducing the plasma glucose level in diabetic rats, thereby preventing excessive production of free radicals through glycation of the proteins. Patra *et al.*<sup>[36]</sup>, studying the effect of aqueous leaf extract (150 mg/kg body wt) on blood gluco-lipid profile in alloxan-induced diabetic rats, showed that *E. globulus* possess hypoglycemic activities with concurrent hypolipidemic effects. They reported that the decrease in blood gluco-lipid profile caused by the aqueous extract of *E. globulus* at a dose 150 mg/kg body wt, is comparable with the effect of sulphonylureas which promote insulin secretion by closure of K<sup>+</sup> ATPase channels, membrane depolarization and stimulation of Ca<sup>2+</sup> ion

influx, an initial key step in insulin secretion. The ability of *E. globulus* to restore body wt in alloxan treated diabetic rats may be due to its hypoglycemic effect while the hypolipidemic effect is due to inhibition of endogenous synthesis of lipids. In alloxan-induced diabetic models, because of metabolic aberration there is a high turnover of triglycerides and phospholipids. *E. globulus* is thought to antagonize this metabolic aberration and restore normal metabolism by tilting the balance from high lipid to high carbohydrate metabolism in alloxan diabetic rats. Patra *et al.*<sup>[36]</sup> showed the antidiabetic activity of the aqueous extract of *E. citriodora* leaf in alloxan-induced diabetic rats. Aqueous extract of leaves of *E. citriodora* exhibited significant antidiabetic activity which was comparable with the standard drug Glibenclamide. Villaseñor *et al.*<sup>[37]</sup>, using an oral glucose tolerance test, showed that *E. tereticornis* exhibits hypoglycemic activity in mice at a dose of 5 mg/20 mg. Experiments carried out by Shahraki *et al.*<sup>[38]</sup> showed that eucalyptus aqueous extract decreased blood glucose level but increased liver enzyme activities in STZ-induced diabetic male rats. The probable hypoglycemic effect may be due to water soluble compounds present in the aqueous extract of eucalyptus which effected on glucose metabolism in fat or skeletal muscle cells and decreased blood sugar by increasing the glucose influx in the cells. Moreover, effects on glycolysis and an increase in glucose consumption in fat and skeletal muscles are also suggested to cause a decrease in blood glucose. Gallagher *et al.*<sup>[39]</sup> investigated the effects of administration of eucalyptus extract on intestinal absorption in rat cultured cells where the extract decreased glucose in the culture environment and increased glucose uptake by the cells. Sugimoto *et al.*<sup>[40]</sup> have shown that *E. globulus* leaf extract (10 g/kg diet) inhibited intestinal fructose absorption and suppressed adiposity due to dietary sucrose in rats. The *E. globulus* leaf extract inhibited intestinal fructose absorption in a dose dependent manner and simultaneously reduced plasma and hepatic triacylglycerol concentrations. The suggested mechanism is that fructose is transported across the intestinal brush border membrane by the specific transporter GLUT5 and inhibiting intestinal fructose absorption prevents adiposity in subjects consuming large amounts of sucrose and fructose. Fructose is metabolized in the liver by fructokinase which is later split by the action of aldolase B into glyceraldehydes and dihydroxyacetone phosphate, the intermediates of glycolytic sequence. Fructose can provide carbon atoms for both glycerol and acyl portions of the TG molecules and is considered to be more lipogenic than glucose. *E. globulus* leaf extract is found to inhibit the activities of fructokinase and G6PDH, preventing the activation of fructose metabolism and fatty acid synthesis induced by dietary sucrose. *E. globulus* leaf extract simultaneously inhibits intestinal fructose and sucrose absorption and shows enough potential to be used as a natural food additive in fructose/sucrose rich junk foods<sup>[40,41]</sup>. Gireesh *et al.*<sup>[42]</sup> showed that incorporation of *E. globulus* in the diet (20 and 62.5 g/kg) and drinking

water (2.5 g/L aqueous extract) of STZ-induced male wistar rats ameliorated their diabetic state in a dose dependent manner with partial restoration of pancreatic  $\beta$ -cells and repair of STZ-induced damage. Thus, this study supports the use of *E. globulus* as an effective anti-hyperglycemic dietary supplement which can, in a dose dependent manner, compensate for STZ-induced cell damage of pancreatic  $\beta$ -cells<sup>[42]</sup>. Studies carried out by Pérez *et al.*<sup>[43]</sup> showed that oral and *ip* administration of eucalyptus extract (25, 50, 75, 100 g per 250 mL water) in alloxan-induced diabetic mice led to hypoglycemia up to 36% (oral) and 25% (*ip*), respectively. Ahlem *et al.*<sup>[44]</sup> showed significant reduction in blood glucose in alloxan-induced diabetic rats with *E. globulus* extract (130 mg/kg body wt) but, since liver glycogen level was not restored, did not recommend the insulin stimulatory effect of eucalyptus, rather highlighting the antioxidative potential of *E. globulus*.

## TOXICOLOGY AND POSOLOGY OF EUCALYPTUS

Limited research data are available on the posology and toxicology of eucalyptus. Shalaby *et al.*<sup>[45]</sup> reported that the essential oil from *E. globulus* in its undiluted form showed an LD<sub>50</sub> value (median lethal dose) of 2334.4 mg/kg body wt and considered it to be moderately hazardous as per WHO specifications. The European Medicines Agency Assessment Report, 2012 on *E. globulus* gave some clinical and non-clinical data on the posology and toxicology of eucalyptus. Other than the traditional use of eucalyptus leaf decoction as herbal tea there is no such specific medicinal product mentioned in any pharmaceutical market overview using eucalyptus leaf or preparation as a single active ingredient. However there are herbal preparations in the literature and monographs on the use of dried leaf infusion or hot infusion mostly as an inhalant, herbal tea, powdered whole drug and aqueous alcoholic extracts or tinctures of varying strengths, or as one of the ingredient of polyherbal preparations mostly for the treatment of cough associated with cold<sup>[46]</sup>. Aboriginal Pharmacopoeias and other earlier citations have recommended use of eucalyptus in treating fever, neuralgic pain, asthma, lung tuberculosis, UTIs, antiseptic, rheumatism, malaria, fevered diarrhea, gum bleeding, anti-helminthic, wound, acne, poorly healing ulcers, gonorrhoea<sup>[7,47]</sup>. Available data on the posology of eucalyptus is highly variable and inconsistent. Nonetheless, according to the European monographs, British Herbal Compendium, German Pharmacopoeia, Spanish literature and literature citations a dosage regimen is described for infusions of from 1.5-3 g of herbal substance for single use and 2.5-20 g for daily use. Posology given by Pharmacopoe Francaise recommends use of herbal tea prepared with 1.5-3 g of eucalyptus leaves in 150 mL of boiling water, steeping time 10-15 min, 4 times a day. For inhalations the posology ranges from 2-3 g per single dose taken up to 3 times a day. For

the oral use of tinctures the posology data widely varies from 1-10 g a day, however the most cited data suggest 2.5 g tincture 1-3 times a day which corresponds to 0.5 g herbal substance as a single dose and 0.5-1.5 g herbal substance a day<sup>[47]</sup>.

Similarly, toxicological data on the eucalyptus are scarce and most reports are on 1,8-cineole which is the main constituent of the essential oil of the eucalyptus species. Hagan *et al*<sup>[46]</sup> reported the LD<sub>50</sub> oral dose to be 2480 mg/kg body wt; De Vincenzi *et al*<sup>[48]</sup> reported it to be 2400 mg/kg per day, although encapsulated cineole showed a dose-related histopathological alteration in liver, kidney and parotid gland at a maximal dose of 5607 mg/kg per day. Kristiansen *et al*<sup>[49]</sup> found that administration of 1,8-cineole at doses 500 and 1000 mg per day for 28 d caused renal lesions in Wister rats. In any case, use of concentrated extract or undiluted oil is not recommended. Pharmacokinetically, 1,8-cineole is very well absorbed orally, topically, or via mucosa and reaches a peak plasma concentration within 1-3 h and is subject to renal and pulmonary excretion. The Commission E monograph cites a number of side effects of eucalyptus following oral administration including allergic skin reaction, shock, tremor, ataxia, aphasia, vomiting, dizziness, urticaria, diarrhea, epigastric pain, and topical reactions such as pustular rash. Co-administration is to be avoided with barbiturates, benzodiazepines, anti-depressants. The side effects can be severe with a number of contraindications so use during pregnancy and lactation is best to be either avoided or only under strict medical supervision since insufficient safety data is available on humans<sup>[46]</sup>.

## CONCLUSION

The detailed chemo-profiling and available literature on the species of eucalyptus discussed in the manuscript, supports their hypoglycemic potential and shows that there is enough promise to use eucalyptus or its bioactive phytochemicals as important therapeutic target against type 2 DM. Species presenting a wide range of phytochemicals need further screening using the latest combinatorial/bioinformatic/computational approaches aided by the latest analytical methodologies which apply hyphenated techniques to the search for novel lead compounds and the mechanistic study of their hypoglycemic potential. However, despite immense potential the popularity of herbal medicines is always hindered due to the traditional ways in which they are delivered, which in many cases not only causes patient non-compliance but also results in reduced efficacy of the drug due to bioavailability problems, organoleptic unacceptability etc. Keeping in mind the hypoglycemic potential of eucalyptus, efforts are needed to develop definitive dosage formulations with eucalyptus bio-actives. Nano-technical approaches can be exploited for their delivery so as to minimize the side effects, bioavailability and organoleptic problems associated with them and, thereby, increase the popularity of using eucalyptus against type 2 DM.

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## Diabetic cardiomyopathy: Pathophysiology, diagnostic evaluation and management

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### Abstract

Diabetes affects every organ in the body and cardiovascular disease accounts for two-thirds of the mortality in the diabetic population. Diabetes-related heart disease occurs in the form of coronary artery disease (CAD), cardiac autonomic neuropathy or diabetic cardiomyopathy (DbCM). The prevalence of cardiac failure is high in the diabetic population and DbCM is a common but underestimated cause of heart failure in diabetes. The pathogenesis of diabetic cardiomyopathy is yet to be clearly defined. Hyperglycemia, dyslipidemia and inflammation are thought to play key roles in the generation of reactive oxygen or nitrogen species which are in turn implicated. The myocardial interstitium undergoes alterations resulting in abnormal contractile function noted in DbCM. In the early stages of the disease diastolic dysfunction is the only abnormality, but systolic dysfunction supervenes in the later stages with impaired left ventricular ejection frac-

tion. Transmitral Doppler echocardiography is usually used to assess diastolic dysfunction, but tissue Doppler Imaging and Cardiac Magnetic Resonance Imaging are being increasingly used recently for early detection of DbCM. The management of DbCM involves improvement in lifestyle, control of glucose and lipid abnormalities, and treatment of hypertension and CAD, if present. The role of vasoactive drugs and antioxidants is being explored. This review discusses the pathophysiology, diagnostic evaluation and management options of DbCM.

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**Key words:** Diabetic cardiomyopathy; Cardiac autonomic neuropathy; Coronary artery disease; Heart failure; Transmitral Doppler Echocardiography

**Core tip:** Cardiovascular disease accounts for most of the diabetes-related morbidity and mortality. Coronary artery disease (CAD), cardiac autonomic neuropathy and diabetic cardiomyopathy (DbCM) are the direct cardiac complications of diabetes. Heart failure risk is two to five times higher in diabetics than in nondiabetics. DbCM is a common, but often unrecognized, complication of diabetic heart disease. Diabetes-induced hyperglycemia, dyslipidemia and inflammation cause damage to the myocardial tissues that result in DbCM. Transmitral Doppler Echocardiography, tissue Doppler Imaging and cardiac Magnetic resonance imaging are used for diagnosis of DbCM. Management of DbCM should target healthy lifestyle, prompt control of diabetes and dyslipidemia, and treatment of hypertension and CAD, if coexistent.

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## INTRODUCTION

Diabetes mellitus affected more than 371 million people worldwide and the global expenditure for healthcare of diabetes in the year 2012 alone was more than 471 billion United States Dollars<sup>[1]</sup>. The disease affects almost every tissue in the body and causes significant organ dysfunction that results in diabetes-related morbidity and mortality. Cardiovascular diseases account for about 65% of diabetes-related mortality and therefore, the American Heart Association (AHA) accepted diabetes as coronary heart disease equivalent towards the turn of the 20<sup>th</sup> century<sup>[2]</sup>.

Diabetes affects the heart in 3 ways: (1) coronary artery disease (CAD) due to accelerated atherosclerosis; (2) cardiac autonomic neuropathy (CAN); and (3) diabetic cardiomyopathy (DbCM). Although there is high awareness among clinicians about the first two disease entities, DbCM is poorly recognized by most physicians and diabetologists. The purpose of this review is to elaborate the pathophysiology, diagnostic evaluation and management options and to highlight the importance of early identification of DbCM to optimize the care of patients with diabetes.

DbCM was first described by Rubler *et al*<sup>[3]</sup> in 1972. DbCM is defined as myocardial dysfunction occurring in patients with diabetes in the absence of CAD, hypertension, or valvular heart disease<sup>[3,4]</sup>. Diabetes is a well-known risk factor for the development of heart failure and the Framingham Heart Study showed that the frequency of heart failure is double in diabetic men and five times in diabetic women compared to age-matched control subjects<sup>[5]</sup>. Heart failure reduces the quality of life of the affected individual and complicates the management of diabetes by alterations in the pharmacokinetics of anti-diabetic medications. Therefore, early diagnosis and prompt management of these patients are of utmost importance.

### Epidemiology

The prevalence of different degrees of heart failure among diabetic subjects was as high as 19%-26% in different major clinical trials<sup>[6-8]</sup>. The actual prevalence of DbCM is not yet established, because of the lack of large study data from different populations with diabetes. The prevalence of diastolic dysfunction in patients with type 2 diabetes mellitus (T2DM) was shown to be up to 30% in some studies<sup>[9,10]</sup>. However, there are other studies which reported a prevalence as high as 40%-60%<sup>[11-13]</sup>. The small numbers of participants in all these studies limit their utility to estimate the true prevalence of the disease in a common disease like diabetes.

A recent major prospective study examining the prevalence of myocardial dysfunction (MD) and heart failure (HF) in patients with longstanding ( $\geq 10$  years) type 1 diabetes mellitus (T1DM) showed a prevalence of 14.5% and 3.7% respectively at the end of a seven year follow up<sup>[14]</sup>. The annual incidence of MD and HF were 0.1% and 0.02% respectively. At baseline, diastolic HF constituted 85% of the cases with HF. Different patient

selection criteria and various techniques of imaging used for diagnosis may explain the disparity in the reported prevalence of DbCM.

### Pathogenesis and pathophysiology

The pathogenesis and pathophysiology of DbCM is not yet fully defined. The development of diabetic cardiomyopathy is multi-factorial. Various proposed mechanisms include metabolic disturbances, insulin resistance, microvascular disease, alterations in the renin-angiotensin system (RAS), cardiac autonomic dysfunction and myocardial fibrosis<sup>[15]</sup>. Chronic hyperglycemia is thought to play a central role in the development of DbCM, although multiple complex mechanisms and interplay of many molecular and metabolic events within the myocardium and plasma contribute to the pathogenesis.

The main metabolic abnormalities in diabetes are hyperglycemia, hyperlipidemia and inflammation, all of which stimulate generation of reactive oxygen or nitrogen species that cause most of the diabetic complications, including diabetic nephropathy and cardiomyopathy<sup>[16,17]</sup>. Several adaptive responses caused by these metabolic abnormalities finally result in cardiac dysfunction and heart failure.

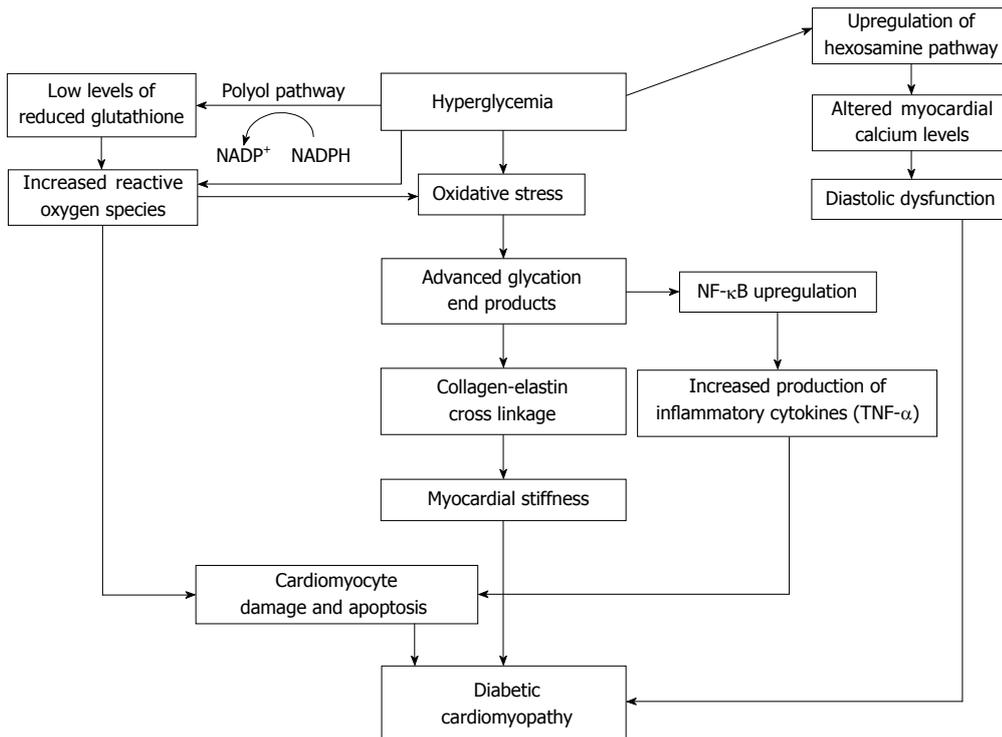
## HYPERGLYCEMIA AND THE HEART

Chronic hyperglycemia results in a number of metabolic and molecular changes in the myocardial cells. Increased glucose metabolism due to hyperglycemia leads to an increase in oxidative stress by generation of reactive oxygen species (ROS) from mitochondria<sup>[18]</sup>. Overproduction of superoxide by the mitochondrial respiratory chain and the consequent oxidative stress result in reduction of myocardial contractility and eventually myocyte fibrosis<sup>[19]</sup>. ROS and oxidative stress can cause cellular DNA damage and acceleration of cardiomyocyte apoptosis.

DNA damage induced by oxidative stress also activates poly ADP ribose polymerase (PARP), a DNA reparative enzyme<sup>[20]</sup>. PARP diverts glucose metabolism from its usual glycolytic pathway (through inhibition of glyceraldehyde phosphate dehydrogenase) into alternative biochemical pathways that result in generation of various mediators which causes hyperglycemia induced cellular injury. These include advanced glycation end products (AGEs), increased flux of hexosamine and polyol, and activation of the enzyme protein kinase C.

Oxidative stress induced by chronic hyperglycemia has been shown to increase the AGEs in diabetic subjects<sup>[19]</sup>. AGEs can covalently crosslink various intra and extracellular proteins that is thought to be a pivotal factor in diabetic complications. The crosslink in collagen and elastin results in increased myocardial stiffness and impaired cardiac relaxation. AGEs are found to induce myocardial damage in both animals<sup>[21]</sup> and human beings<sup>[22]</sup>.

AGEs also indirectly exert their detrimental effect on the myocardium by interacting and up-regulating their



**Figure 1 Mechanism of myocardial damage resulting from hyperglycemia.** NADPH: nicotinic acid adenine dinucleotide phosphate; NF- $\kappa$ B: Nuclear factor- $\kappa$ B; TNF- $\alpha$ : Tumour necrosis factor- $\alpha$ .

receptors, including receptors of AGE and galectin-3<sup>[21]</sup>. This results in activation of transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B). NF- $\kappa$ B dependent genes in turn trigger several pathways that induce production of pro-inflammatory cytokines such as Tumour necrosis factor- $\alpha$  and cause myocardial damage<sup>[23]</sup>. NF- $\kappa$ B blockers were found to attenuate mitochondrial oxidative stress and protect against cardiac dysfunction in diabetic mice<sup>[24]</sup>.

Chronic hyperglycemia can lead to increased flux of glucose into the alternate metabolic pathway known as hexosamine pathway that is implicated in many adverse consequences of diabetes. Increased glucose metabolism in the hexosamine pathway is associated with disruption of normal cardiomyocyte calcium flux linked to reduced sequestration of calcium in the sarcoplasmic reticulum<sup>[25]</sup>. This results in reduction in myocardial performance and impaired diastolic relaxation, a possible mechanism for DbCM.

Polyol pathway is also activated by chronic hyperglycemia and glucose is converted to sorbitol by the action of the enzyme aldose reductase in the presence of nicotinic acid adenine dinucleotide phosphate (NADPH) that is oxidized to NADP<sup>+</sup>. NADPH is a co-factor essential for regeneration of reduced glutathione, an important scavenger of ROS in the body, and increased utilization of NADPH in the polyol pathway disturbs the redox balance of cells. The consequent increase in oxidative stress can lead on to DNA damage and cardiomyocyte apoptosis<sup>[26]</sup>. Sorbitol can also glycate proteins that results in formation of AGEs, which are mediators of tissue injury in diabetes<sup>[27,28]</sup>. A diagrammatic representa-

tion of cardiac damage resulting from hyperglycemia is shown in the Figure 1.

## LIPID METABOLISM AND THE MYOCARDIUM

Overstrain of cellular oxidation capacity in diabetes leads on to ectopic lipid deposition in non-adipose tissues such as skeletal muscle, liver and heart. Cardiac steatosis (increased myocardial lipid content), resulting from disturbed myocardial substrate metabolism, has been proposed as an important cause for DbCM recently<sup>[29-31]</sup>. Hyperinsulinemia, hyperglycemia and elevated levels of plasma free fatty acids (FFA) are the classical metabolic abnormalities in T2DM that lead on to cardiac steatosis. Patients with diabetes, obesity and impaired glucose tolerance were found to have cardiac steatosis of varying degrees<sup>[29,30,32]</sup>.

The contribution of glucose oxidation to cardiac energetics is less than normal among patients with obesity and T2DM and fatty acid metabolism is enhanced to meet the myocardial energy needs<sup>[33,34]</sup>. Increased plasma FFA levels in patients with T2DM and obesity, result in increased cardiac fatty acid (FA) uptake and triglyceride accumulation. Excessive FA delivery and uptake by cardiomyocytes in this setting is likely to exceed mitochondrial oxidative capacity and consequently leads on to lipotoxic cardiac injury. Part of the excess FA enters nonoxidative pathways, giving rise to toxic FA intermediates such as ceramide. These toxic substances in turn

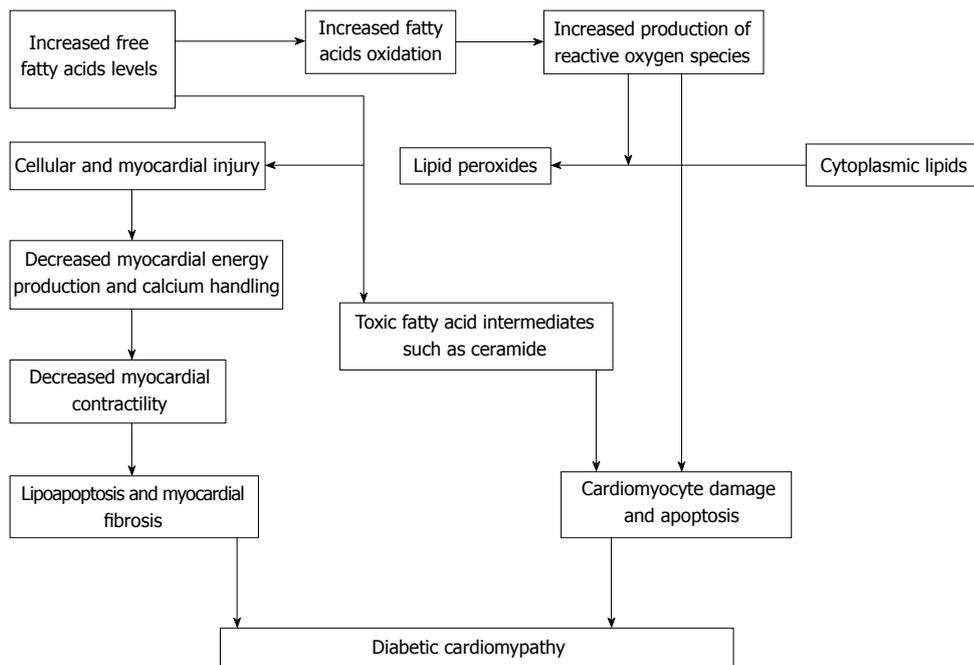


Figure 2 lipotoxic cardiac injury in diabetic cardiomyopathy.

disrupt normal cellular signaling and cause mitochondrial dysfunction, cellular damage, apoptosis, and eventually myocardial fibrosis and contractile dysfunction.

Although some earlier studies showed predominantly diastolic dysfunction in diabetic patients with cardiac steatosis<sup>[29,30]</sup>, recent evidence demonstrated biventricular systolic and diastolic dysfunction in patients with high myocardial triglyceride levels<sup>[35]</sup>. Intracellular accumulation of triglyceride alone is unlikely to be the cause of cardiac injury as it is relatively inert. However, the intermediate metabolites derived from nonoxidative pathways of intracellular lipid handling are probably responsible for lipotoxic tissue injury and eventual cellular apoptosis<sup>[36]</sup>.

Increased FA oxidation in the mitochondria is associated with an increase in generation of ROS that oxidizes cytoplasmic lipids into lipid peroxides. ROS and lipid peroxides in turn cause cellular and mitochondrial damage and uncoupling of mitochondrial oxidative metabolism<sup>[37]</sup>. Consequently, impaired myocardial generation of energy and reduced cardiac contractility results. Decreased production of energy also leads to an impaired mitochondrial calcium handling that causes cardiac dysfunction<sup>[38]</sup>.

Cell apoptosis that results from lipotoxicity is commonly referred to as lipoapoptosis. Different mechanisms such as palmitate toxicity, ceramide and diacylglycerol formation, endoplasmic reticulum stress, membrane destabilization and inflammation may result in lipoapoptosis<sup>[37]</sup>. Structural damage and myocardial fibrosis are the results of lipoapoptosis that compromise the cardiac function. Figure 2 shows a model of lipotoxic cardiac injury.

Elevated levels of plasma FFA also induce cellular insulin resistance by various mechanisms<sup>[39]</sup>. These include activation of protein kinase C (PKC), and peroxisome

proliferator-activated receptor-gamma and  $\alpha$  (PPAR- $\gamma$  and PPAR- $\alpha$ ). PKC is a family of several isoenzymes that regulates various complex cellular metabolic pathways, and is thought to play a key role in generation of insulin resistance. Similarly, the activation of PPAR- $\gamma$  and PPAR- $\alpha$  also results in hyperinsulinemia and insulin resistance, mediated through different complex mechanisms.

## ROLE OF HYPERINSULINEMIA AND INSULIN RESISTANCE

Hyperinsulinemia and insulin resistance are the characteristic pathological abnormalities in T2DM and prediabetic states. Hyperinsulinemia results in cardiomyocyte hypertrophy by various mechanisms. Brain natriuretic peptide (BNP), a biomolecule released from the ventricles in response to myocardial stretch, has been found to be increased in patients with heart failure. BNP is also an important molecular marker of cardiac hypertrophy. BNP gene expression was found to be significantly higher among animal models of hyperinsulinemia and insulin resistance<sup>[40]</sup>. Left ventricular hypertrophy and increased left ventricular weight were also found in these animal models. Recently, BNP has emerged as a useful biomarker for screening subclinical ventricular diastolic dysfunction in patients with uncontrolled diabetes<sup>[40,41]</sup>.

Hypertrophy of cardiac myocytes in diabetes was found to be regulated at the transcriptional level<sup>[42]</sup>. Various genetic and epigenetic alterations resulting from hyperinsulinemia, leads on to activation of multiple transcription factors that modulate cellular and extracellular protein expression. Activation of such transcription factors have been shown to result in cardiomyocyte hypertrophy and deposition of extracellular matrix proteins

causing focal cardiac fibrosis in diabetes<sup>[42,43]</sup>.

## CONTRIBUTION FROM MICROVASCULAR ISCHEMIA

The pathological hallmark of diabetes-related vascular complications is damage to the microvasculature throughout the body. Classical examples of microvascular complications are diabetic retinopathy, nephropathy and neuropathy. Hyperglycemia confounded by other factors such as hypertension, lipid abnormalities and smoking impose oxidative stress on the vascular endothelium that leads on to endothelial dysfunction, the earliest abnormality in patients with diabetes. Nitric oxide (an endothelium-derived vasodilatory factor) production in relation to vascular stretch is also reduced due to down regulation of endothelial nitric oxide synthase enzyme in diabetes<sup>[44,45]</sup>.

Hyaline change (amorphous, ground-glass appearance resulting from breakdown of structural proteins like collagen) of the medial layers of arterioles and reduction of capillary length density throughout the cardiac circulation is seen in diabetics<sup>[15,46]</sup>. The reduced blood supply resulting from microvascular disease affecting the vasa vasorum in diabetes, further damages the small and medium arterioles of the diabetic heart. Thickening of the capillary basement membrane, formation of microaneurysms in small vessels, perivascular fibrosis and interstitial changes are the other vascular abnormalities causing cardiac microvascular ischemia in diabetes. Ischemia contributes to myocardial stiffness, fibrosis and cardiac dysfunction in DbCM.

## ROLE OF RAS

Recent evidence from animal and human experiments have demonstrated significant role of RAS in diabetes-induced cardiac dysfunction<sup>[47-49]</sup>. All major components of the classical RAS, *i.e.*, renin, angiotensinogen, angiotensin converting enzyme (ACE), angiotensin II (AGT II) receptors are expressed in the heart<sup>[48]</sup>. Hyperglycemia activates intra-cardiac RAS that has various effects on the myocardial cells. Intracellular AGT II levels were found to be 3.4-fold higher in the cardiomyocytes of diabetic patients compared to nondiabetics<sup>[50]</sup>.

Cytoplasmic AGT II has been shown to induce cell growth in animal models. AGT II has a direct effect on cell signaling that results in hypertrophy in cardiac myocytes and proliferation of cardiac fibroblasts<sup>[48]</sup>. Other factors, such as oxidative stress, inflammation and aldosterone, may contribute to the deleterious effects of AGT II on the heart producing myocardial damage in diabetes<sup>[49]</sup>.

## CARDIAC AUTONOMIC NEUROPATHY AND DBCM

Cardiac autonomic neuropathy (CAN) is a common

complication of longstanding diabetes that causes abnormalities in heart rate control and vascular hemodynamics. The prevalence of varying degrees of CAN may be as high as 60% in individuals with prolonged history of diabetes<sup>[51]</sup>. CAN affects blood flow in the coronary vasculature and also alters the contractile function of the myocardium. Patients with CAN were found to have a reduction in the vascular elasticity and an increase of peripheral vascular resistance due to abnormal sympathetic tone<sup>[52]</sup>. Reduction in myocardial perfusion reserve also was shown by other investigators<sup>[53]</sup>. This may partly explain the ventricular dysfunction associated with diabetic CAN.

Ventricular dysfunction was found to be common in diabetic patients with CAN<sup>[15,54]</sup>. Correlation between the severity of CAN and the prevalence of diastolic dysfunction also have been demonstrated<sup>[55]</sup>. Alterations in the myocardial contractility responses in relation to stress is seen in patients with diabetic CAN, and even in those with normal ventricular function at rest, exercise-induced myocardial dysfunction have been demonstrated<sup>[56,57]</sup>.

## STRUCTURAL AND FUNCTIONAL ALTERATIONS IN DBCM

Significant changes in the anatomy and the function of myocardium occur as a result of DbCM that cause the clinico-pathological consequences of the disease. Pathological alterations occur mainly in the myocardial interstitium (formation of AGEs, impaired compliance and ischemia from the disease in the vasa vasorum) in the early stages and myocardial contractile dysfunction results as a consequence of the above changes<sup>[58]</sup>. Ventricular myocardial hypertrophy, interstitial and perivascular fibrosis and cardiac microvascular abnormalities ensue later.

Impaired diastolic function is the earliest abnormality in DbCM and systolic dysfunction supervenes only at later stages of the disease<sup>[40,41,58,59]</sup>. Diastolic dysfunction is characterized by impaired relaxation of the ventricular musculature during diastole of cardiac cycle and the resultant increase in ventricular filling pressure and diastolic heart failure. Ventricular hypertrophy and fibrosis caused by DbCM are the main reasons for diastolic dysfunction. When systolic dysfunction supervenes the cardiac output diminishes progressively with the severity of disease. LV systolic ejection fraction gives a good reflection of the severity of systolic dysfunction and heart failure.

### **Interaction with coexistent hypertension and CAD**

DbCM is diagnosed only when hypertension and CAD are excluded. However, when these diseases superimpose on existing DbCM, rapid progression to advanced heart failure may result. It is difficult to identify the role of these diseases in the development and progression of DbCM from a clinical perspective. Clinically silent CAD

may further complicate the diagnostic evaluation.

Coexistent hypertension was found in approximately 30% of patients with T1DM and in 50% to 80% of patients with T2DM in the United States<sup>[60]</sup>. Cardiac dysfunction was shown to be worsened by hypertension in animal models of DbCM<sup>[61]</sup>. Presence of hypertension has also been shown to be independently associated with diastolic dysfunction in diabetic patients<sup>[62]</sup>. Similarly, CAD was found to cause myocardial structural abnormalities in diabetic patients<sup>[46]</sup>. Patients with coexistent diabetes and hypertension have a higher incidence of CAD that may worsen myocardial dysfunction.

### Cardiac remodeling in DbCM

DbCM results from the structural, functional and regulatory remodeling of the heart induced by diabetes mellitus. Different stages of remodeling has been proposed: the early stage, middle stage and the late stage<sup>[39]</sup>. The early stage is usually asymptomatic with myocardial changes mostly at the molecular level. Ventricular hypertrophy and diastolic dysfunction with normal left ventricular ejection fraction are the only gross abnormalities demonstrable at this stage.

The middle stage of DbCM is characterized by progressive cardiomyocyte hypertrophy and myocyte fibrosis. Increasing ventricular wall thickness and muscle mass at this stage result in worsening of the diastolic dysfunction and the development of mild systolic dysfunction. Further progression of the disease in the late stage is associated with abnormalities like CAN, microvascular/macrovascular CAD, hypertension, and overt diastolic and systolic dysfunction.

### Diagnostic evaluation of DbCM

A majority of the cases of DbCM are subclinical and the patients may not have any overt symptoms or signs of the disease. In the early stages, there are only substructural changes in the cardiomyocytes, and the detection is possible only by very sensitive methods such as strain, strain rate and myocardial tissue velocity<sup>[15]</sup>. Later on, myocyte hypertrophy and fibrosis develop, that may be associated with structural changes like LV hypertrophy and increased muscle mass (the middle stage of DbCM). Conventional diagnostic methods such as echocardiography may detect diastolic and/or systolic dysfunction at this stage. Significant microvascular changes and fibrosis occurs in the myocardium in advanced stages of DbCM and this stage is usually associated with hypertension, overt heart failure and ischemic heart disease<sup>[15]</sup>.

## ECHOCARDIOGRAPHY

Echocardiography is a relatively inexpensive diagnostic tool for detecting structural and functional cardiac abnormalities. Transmitral Doppler (Mitral valve blood flow measured by pulsed wave Doppler) is the usual technique for assessment of the ventricular diastolic function<sup>[63]</sup>. The variables measured by transmitral Dop-

pler are: the early ventricular filling wave (E-wave) and the late ventricular filling wave (A-wave), which can be reported as the E/A ratio, the isovolumetric relaxation time (IVRT), E-wave peak velocity (E), E-wave deceleration time (EDT) and A-wave duration (A-dur). Based on the above study results, diastolic function can be categorized as: (1) normal pattern; (2) grade I (impaired relaxation); (3) grade II (pseudonormal pattern); and (4) grade III (restrictive pattern)<sup>[64]</sup>.

Patients with grade I diastolic dysfunction (impaired relaxation) show an E/A ratio < 1 that results from a decreased early and increased late diastolic flows<sup>[65]</sup>. An increase in the IVRT and EDT are seen in these subjects<sup>[66]</sup>. In those with grade II diastolic dysfunction (pseudonormal pattern) an E/A ratio > 1 resulting from an increase in left atrial pressure is seen due to defective LV relaxation<sup>[64]</sup>. An increase in filling pressures in order to maintain normal cardiac output is the end result of impaired LV relaxation<sup>[65,67]</sup>. An E/A ratio > 2 is characteristic of grade III (restrictive pattern) diastolic dysfunction, the advanced diastolic heart failure<sup>[64]</sup>. Color M-mode Doppler echocardiography also may be useful to evaluate LV relaxation<sup>[68]</sup>. However, low sensitivity and specificity limits the diagnostic utility of Doppler and M-mode echocardiography in DbCM.

Tissue Doppler imaging (TDI) measures myocardial tissue velocities during the cardiac cycle and can be used to quantitatively assess global and regional systolic and diastolic functions of the myocardium<sup>[69,70]</sup>. TDI is a more sensitive and specific tool for the diagnosis of DbCM compared to the transmitral Doppler technique<sup>[71]</sup>. Newer echocardiographic imaging techniques are evolving with better sensitivities and specificities than these modalities of imaging.

## MAGNETIC RESONANCE IMAGING

Cardiac magnetic resonance imaging (MRI) has recently emerged as a very good imaging tool for the diagnosis of various structural and functional disorders of the myocardium<sup>[72,73]</sup>. Gadolinium-enhanced cardiac MRI have been found to be useful to predict major adverse cardiac events, such as acute myocardial infarction, development of heart failure and ventricular arrhythmias in diabetic patients without previous history of ischemic heart disease<sup>[74]</sup>. Cardiac MRI is also useful to detect diastolic dysfunction and myocardial steatosis<sup>[30]</sup>. Cardiac MRI using different radionuclides and positron emission tomography (PET) can detect myocardial metabolic abnormalities and are the newer imaging techniques that may be useful in the diagnosis of DbCM.

## CARDIAC CATHETERIZATION AND CORONARY ANGIOGRAPHY

Cardiac catheterization is the best method to assess the hemodynamic events within the heart chambers. Diastolic dysfunction documented invasively through cath-

eterization continues to be the most definitive evidence of diastolic heart failure<sup>[75]</sup>. Left ventricular end-diastolic pressure of > 16 mmHg or mean pulmonary capillary wedge pressure > 12 mmHg, determined invasively by catheterization, are the most diagnostic features of diastolic dysfunction<sup>[75]</sup>. However, catheter-based diagnosis of DbCM is rarely used at present because of the availability of noninvasive techniques with high sensitivity and specificity. Coronary angiography is useful for the diagnosis of CAD that may coexist/complicate DbCM. Microvascular CAD is also diagnosed by angiography wherein, the patient presents with symptoms of CAD with normal angiogram. Newer radionuclide-based techniques and CT scan are the noninvasive techniques for coronary evaluation that can be used to diagnose CAD in patients with DbCM.

## SEROLOGIC CARDIAC MARKERS

Changes in the levels of various plasma/serum cardiac biomarkers may reflect some of the myocardial metabolic and structural functions. Strong correlation between turnover of extracellular matrix proteins and ongoing cardiac remodeling has been identified in different studies<sup>[76,77]</sup>. Matrix metalloproteinases (MMPs) are the enzymes that degrade extracellular matrix, increase matrix turn over and alter the expression of several micro-Ribonucleic Acids (mi-RNAs) that lead to contractile dysfunction of the myocardium<sup>[39]</sup>. Elevated levels of MMPs especially MMP9, and reduced levels of the tissue inhibitors of MMPs are seen in myocardial fibrosis. The clinical utility of these novel biomolecules for diagnosis of DbCM is under investigation.

Serum aminoterminal propeptide of type III (PIIINP), an indicator of type III collagen turnover in the body, was suggested to be an early indicator of LV dysfunction in obese subjects with insulin resistance<sup>[78]</sup>. The role of BNP has already been discussed. Epshteyn *et al.*<sup>[79]</sup> showed a high positive predictive value of 96% for plasma BNP levels (> 90 pg/mL) in diabetic subjects for the detection of LV dysfunction with echocardiographic correlation.

Cardiac troponins (T, N and I) are the molecules released to circulation from the injured myocardium from ischemia or inflammatory disease. Elevated troponin T levels were found in infants with cardiac dysfunction and cardiomyopathy born to diabetic mothers<sup>[80]</sup>. However, the role of troponins for evaluation of adult patients with DbCM is not yet clear.

miRNAs are small non-coding RNA molecules that modulate cellular gene expression. The dysregulation of miRNA has been linked to diabetes and many of its complications. Altered levels of miRNAs were observed in the cardiomyocytes of experimental diabetes models<sup>[81]</sup>. These novel molecules may emerge as diagnostic and prognostic tools in the future for patients with DbCM.

## Therapeutic strategies for the management of DbCM

Better understanding of the disease pathogenesis and

pathophysiology in the recent years provides us with improved management options for patients with DbCM. These include changes in lifestyle, improving diabetic control, lipid lowering therapy, management of coexistent hypertension and CAD if present, and management of heart failure.

## LIFESTYLE MEASURES

Regular physical activity and healthy eating habits are two cornerstones of the management of diabetes, especially in the background of the global epidemic of obesity and overweight. Physical activity was associated with significant reduction of all-cause mortality and cardiovascular disease in patients with diabetes in many clinical studies<sup>[82]</sup>. Exercise training has been shown to be beneficial in reducing the incidence of DbCM in both animal models and human subjects<sup>[83-85]</sup>. It is difficult to predict the benefit of physical activity in established cases of the disease in the absence of controlled clinical trials. However, better diabetic control with regular exercise would have beneficial effect on the disease outcome. Healthy eating pattern appropriate for the diabetic individual is also expected to provide similar beneficial effects.

## MANAGEMENT OF DIABETES

Improvement of glycemic control (with HbA1c between 42-53 mmol/mol) has been shown to be associated with better outcomes in diabetic microvascular complications in many clinical trials. However, beneficial effects of strict glycemic control on macrovascular outcomes are still not very clear. Because microvascular disease has important pathogenic role in the development of DbCM, better glycemic control would be expected to benefit patients.

Poor glycemic control in diabetes is associated with increased plasma levels of FFA that cause worsening of the oxidative stress, synthesis of various growth factors and derangement of lipid metabolism, and creates a favorable metabolic and biochemical environment in the body for the development of DbCM. Therefore, optimal diabetic control might be the best and most important strategy for the prevention and treatment of the disease. Better glycemic control has shown to retard DbCM in animal models<sup>[86]</sup>. Tight glycemic control has been shown to improve stress-induced ventricular dysfunction without CAD (possibly DbCM) in poorly controlled diabetic patients in a large prospective study<sup>[87]</sup>. Another case-controlled study of cardiac MRI of patients with T1DM showed that strict glycemic control was associated with better parameters of outcome in DbCM<sup>[88]</sup>. Diabetes management has also been shown to be beneficial in reducing myocardial steatosis<sup>[89]</sup>.

## Insulin

Insulin administration is the cornerstone of management of patients with T1DM, and those with advanced T2DM when other medications fail to control hyper-

glycemia. Prompt administration of insulin, targeting optimal glycemic control, may ameliorate progression of DbCM in established cases.

### **Metformin**

Metformin is believed to be the most widely prescribed anti-diabetic medication in the world. It improves peripheral insulin sensitivity and reduces hepatic glucose output, and thus helps in controlling hyperglycemia. Metformin has shown to upregulate cardiomyocyte autophagy that has role in prevention of diabetic cardiomyopathy in animal models<sup>[90]</sup>. However, there is no data on its role in human beings with DbCM.

### **Pioglitazone**

Pioglitazone increases insulin sensitivity and is used as a hypoglycemic agent in patients with T2DM for nearly 2 decades. The drug is generally not recommended in patients with heart failure because of its propensity to cause fluid retention. Pioglitazone was found to have anti-inflammatory effects that ameliorate cardiac fibrosis in animal models and the drug may prevent the development of DbCM<sup>[91]</sup>.

### **Glucagon-like Peptide-1 mimetics**

Glucagon-like Peptide-1 (GLP-1) is a peptide hormone, secreted by the L-cells of jejunum and ileum of the small intestine, that stimulates meal-related endogenous insulin secretion. Natural GLP-1 has a very short biological half life. Synthetic GLP-1 mimetic agents with longer half-lives such as Exenatide and liraglutide are the new anti-diabetic agents widely used now. Their use in obese T2DM patients is associated with significant improvement in glycemic control and weight loss. GLP-1 agonists has shown to attenuate cardiomyocyte apoptosis in rat models<sup>[92]</sup>. This novel group of drugs may emerge as a promising treatment option in obese T2DM patients with DbCM.

### **Dipeptidyl peptidase-4 inhibitors**

Dipeptidyl peptidase-4 (DPP-4) is an enzyme that metabolizes endogenous GLP-1. DPP-4 inhibitors were shown to prolong the effect of natural GLP-1 produced in the body. Agents in this class, such as sitagliptin, linagliptin, saxagliptin and vildagliptin, are used as effective anti-diabetic medications now. They are weight-neutral and are useful especially in overweight and obese diabetics in combination with conventional anti-diabetic agents like metformin. Use of sitagliptin has been shown to improve myocardial glucose uptake in patients with non-ischemic cardiomyopathy<sup>[93]</sup>. The possible therapeutic role of DPP-4 inhibitors in patients with DbCM is yet to be defined.

### **Amylin analogues**

These novel agents, when administered with insulin, have shown to reduce body weight, HbA1c values and even the insulin requirement<sup>[94]</sup>. Their role in controlling

hyperglycemia may benefit patients with DbCM.

### **Various anti-diabetic combinations**

Achievement of optimal long-term glycemic control with a single anti-diabetic agent is rarely possible in patients with T2DM. Different drug combinations like metformin plus DPP-4 inhibitors/GLP-1 mimetics, metformin plus pioglitazone, metformin plus sulphonylurea, and metformin plus insulin may be necessary for optimal glycemic control. Therefore, appropriate combination anti-diabetic therapy should be chosen, according to the clinical situation, for managing the patients with DbCM.

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## **LIPID LOWERING THERAPY**

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Lipid abnormalities are more harmful in diabetics than in non-diabetic individuals because of their higher atherogenic potential. The particle size of low-density lipoprotein (LDL) cholesterol is smaller in diabetic individuals and this is more atherogenic even with near normal plasma levels. Statin treatment has shown to reduce cardiovascular events and mortality in patients with diabetes and vascular risk factors in multiple clinical trials<sup>[95]</sup>, and are useful even for primary prevention in patients without established cardiovascular disease<sup>[96]</sup>. The vascular remodeling capacity of statins is referred to as the pleiotropic effect.

Atorvastatin, independent of its LDL cholesterol-lowering capacity, has shown to reduce intramyocardial inflammation and myocardial fibrosis, and improve LV function in rat models of experimental DbCM<sup>[97]</sup>. Similarly, fluvastatin also has been shown to be beneficial in attenuating myocardial interstitial fibrosis and cardiac dysfunction in rat models of the disease<sup>[98]</sup>. Although there are no clinical trials investigating the role of lipid lowering therapy in human subjects with established DbCM, beneficial effects of the treatment of dyslipidemia can be anticipated in these patients along with a role in the primary prevention of the disease.

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## **ROLE OF VASOACTIVE DRUGS**

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Various vasoactive medications have been tried in both animal models and human subjects with DbCM with variable results. The most studied ones were those active on the renin-angiotensin systems. Production of angiotensin II within the myocardium has been proposed as a mechanism for the development of DbCM. Aliskiren (inhibitor of renin), benazeprilat (angiotensin convertase enzyme inhibitor; ACEI) and valsartan (angiotensin II receptor blocker; ARB) have all been shown to be protective against DbCM in rat models recently<sup>[99]</sup>. ACEI's and ARB's were also found to be beneficial in both animal and human models of DbCM<sup>[100,101]</sup>.

Beta adreno-receptor blockers were found to be effective in experimental models of DbCM<sup>[102-104]</sup>. Because of the proven beneficial effects of beta-blockers in chronic heart failure, this group of drugs should be con-

sidered for treatment of DbCM, although there are no reported randomized clinical trials examining the benefit of the same. They can be used as effective antihypertensive agents in DbCM cases with high blood pressure. Similarly, calcium channel antagonists were also found to be beneficial in animal models of DbCM<sup>[105-107]</sup>. However, data on human subjects is lacking, to make evidence-based recommendations for the use of these agents in the management of DbCM, especially in the absence of coexistent hypertension.

Sildenafil, a selective phosphodiesterase type 5 inhibitor, has recently been shown to improve cardiac remodeling, myocardial function and few circulatory markers of cardiac inflammation in patients with DbCM<sup>[108]</sup>. Larger clinical trials in the future may prove if this novel agent can be recommended routinely in patients with the disease.

## ROLE OF ANTIOXIDANTS

Trimetazidine is an atypical anti-anginal agent with antioxidant properties that shifts cardiac energy metabolism from free fatty acid oxidation to glucose oxidation. The drug has shown promising beneficial effect on heart failure in diabetic patients with both ischemic and idiopathic dilated cardiomyopathy<sup>[109,110]</sup>. Animal model has shown that trimetazidine improved myocardial function by attenuating lipotoxicity and augmented oxidation status of the heart and might suppress the development of DbCM<sup>[111]</sup>. Human trials are needed to investigate the beneficial effects of this well-tolerated drug on treatment and prevention of DbCM.

Although experimental models have shown beneficial effects of alpha-lipoic acid on cardiac redox homeostasis and suppression of cardiac fibrosis<sup>[112]</sup>, human data is not yet available. Other investigational agents, found to be useful recently in animal models are, riboflavin<sup>[113]</sup>, luteolin<sup>[114]</sup>, sodium ferulate<sup>[115]</sup> and resveratrol<sup>[116]</sup>. Many other agents that have been tried in lab animals are not discussed here as they are beyond the scope of this review.

## EMERGING TREATMENT MODALITIES

The dysregulation of miRNA function being an important pathogenic mechanism of diabetes and its complications like DbCM, artificial restoration of normal function can be a potential therapeutic target. Specific miRNA targets are found useful in the treatment of structural heart disease in mice<sup>[117]</sup>. Similarly, transplantation of bone marrow-derived endothelial progenitor cells (stem cell therapy) has shown to ameliorate DbCM in rat models<sup>[118]</sup>. Ongoing research may help us to translate the success in these experimental models to clinical practice in the future.

## MANAGEMENT OF COEXISTENT HYPERTENSION, CAD AND HEART FAILURE

There are no formal guidelines on the management of

coexistent hypertension and cardiac ischemia in patients with DbCM. However, when these diseases coexist, they accelerate the progression of DbCM because of their detrimental effects on ventricular function and structure. Optimal treatment of hypertension and CAD would be expected to ameliorate the disease progression and even slow it down. Coronary intervention in appropriate cases with significant CAD may improve the symptoms and clinical outcomes. Management of heart failure depends on the type (diastolic or systolic), severity and associated conditions like hypertension and CAD.

## CONCLUSION

DbCM is an important but less well-recognized complication of longstanding diabetes that is associated with significant cardiac morbidity and mortality. The wide disparity in the reported prevalence of the disease may be related to differences in the types of diagnostic tests used by various investigators. The pathogenesis and pathophysiology of DbCM are still not fully elucidated, although suggested pathogenic mechanisms include chronic hyperglycemia-associated oxidative and metabolic stress, lipotoxicity, insulin resistance, microvascular disease, CAN and coexistent hypertension and CAD. The disease manifestations can vary from sub-clinical ventricular dysfunction to overt heart failure. Echocardiography is the standard clinical diagnostic tool for DbCM at present. Newer investigative modalities like cardiac MRI, radionuclide scans, PET imaging and various plasma markers are emerging in the diagnostic armamentarium. The management of DbCM includes changes in lifestyle, good glycemic control, treatment of dyslipidemia, coexistent hypertension, CAD and medications for heart failure.

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## Effects of glucagon-like peptide-1 receptor agonists on renal function

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### Abstract

Glucagon-like peptide-1 (GLP-1) receptor agonists result in greater improvements in glycemic control than placebo and promote weight loss with minimal hypoglycemia in patients with type 2 diabetes mellitus. A number of case reports show an association of GLP-1 receptor agonists, mainly exenatide, with the development of acute kidney injury. The present review aims to present the available data regarding the effects of GLP-1 receptor agonists on renal function, their use in subjects with chronic renal failure and their possible association with acute kidney injury. Based on the current evidence, exenatide is eliminated by renal mechanisms and should not be given in patients with severe renal impairment or end stage renal disease. Liraglutide is not eliminated by renal or hepatic mechanisms, but it should be used with caution since there are only limited data in patients with renal or hepatic impairment. There is evidence from animal studies that GLP-1 receptor agonists exert protective role in diabetic nephropathy with mechanisms that seem to be independent of their glucose-lowering effect. Additionally, there is evidence that GLP-1 receptor agonists influence water and electrolyte balance. These effects may represent new ways to improve or even prevent diabetic nephropathy.

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**Key words:** Glucagon-like peptide 1; Glucagon-like peptide 1 receptor agonists; Exenatide; Liraglutide; Kidney; Renal impairment; Diabetic nephropathy; Electrolytes

**Core tip:** Glucagon-like peptide-1 (GLP-1) receptor agonists improve glycemic control in patients with type 2 diabetes mellitus. A number of case reports show an association of GLP-1 receptor agonists, mainly exenatide, with the development of acute kidney injury. Exenatide is eliminated by renal mechanisms, but liraglutide is not eliminated by renal or hepatic mechanisms. GLP-1 receptor agonists exert protective role in animal models of diabetic nephropathy. The effects of these drugs may represent new ways to improve or even prevent diabetic nephropathy, but their exact mechanism of action need to be elucidated.

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### INTRODUCTION

Increased glucose states are associated with many cardiovascular and renal complications<sup>[1-5]</sup>. Furthermore, the incidence of type 2 diabetes mellitus (T2DM) is increasing dramatically and is associated with high morbidity and mortality rates<sup>[6,7]</sup>. Various drug therapies are used in the treatment of T2DM and its complications<sup>[8,9]</sup>. Recent evidence has demonstrated the beneficial effects of glucagon-like peptide-1 (GLP-1)-associated drugs in the treatment of T2DM<sup>[10]</sup>.

GLP-1 is an incretin hormone secreted by the small intestine in response to nutrient ingestion and degraded

by the enzyme dipeptidyl peptidase-IV (DPP-IV). GLP-1 acts through the GLP-1 receptor, which is a G-coupled protein receptor expressed in the gastrointestinal tract, but also in the nervous system, heart, vascular smooth muscles, proximal tubules and glomerulus of the kidney<sup>[11-13]</sup>. GLP-1 increases insulin secretion from pancreatic  $\beta$ -cells and reduces glucagon release from  $\alpha$ -cells through induction of adenylate cyclase and cyclic adenosine monophosphate (cAMP) production<sup>[14,15]</sup>. GLP-1 also decreases gastric motility and emptying and increases the feeling of satiety<sup>[16,17]</sup>. GLP-1 has been associated with modulation of cardiovascular risk factors and atherosclerosis-related mechanisms, as well as with cardiomyocyte and neuronal cell protection<sup>[14,18,19]</sup>.

GLP-1 receptor agonists extend the effects of endogenous GLP-1 by resisting enzymatic degradation<sup>[20]</sup>. The GLP-1 receptor agonist exendin-4 is a 39-amino acid peptide that was originally isolated from the salivary secretions of the Gila monster lizard<sup>[21]</sup>. It shares approximately 53% homology with the mammalian GLP-1, binds to the mammalian receptor and activates it for synthesis of GLP-1. A synthetic version of exendin-4, exenatide, is currently used for the treatment of T2DM. The extended activity of liraglutide, the second GLP-1 receptor agonist used for the treatment of T2DM, is due to structural modifications of the human GLP-1 peptide aiming to increase its circulating time<sup>[20]</sup>.

The administration of GLP-1 receptor agonists results in greater improvements in glycemic control than placebo when administered as monotherapy or in combination with one or two oral antidiabetic drugs in patients with T2DM. Moreover, these drugs promote weight loss with minimal hypoglycemia and seem to exert a number of other pleiotropic effects on cardiovascular complications of T2DM and diabetic nephropathy<sup>[22-25]</sup>. However, there are concerns regarding the long-term consequences of incretin-associated therapies, which are focused on the lack of evidence on long-term cardiovascular effects and issues raised regarding possible side effects, such as the development of acute pancreatitis, chronic pancreatitis, pancreatic cancer and thyroid cancer<sup>[26-30]</sup>. Furthermore, there are a number of reports associating the use of these drugs with the development of acute kidney injury<sup>[31,32]</sup>.

The present review aims to present the available data regarding the effects of GLP-1 receptor agonists on renal function, their use in subjects with chronic renal failure and their possible association with acute kidney injury.

A PubMed/Scopus search was performed up to June 2013 using combinations of “glucagon-like peptide-1 receptor agonists” with the following keywords: exendin-4, exenatide, liraglutide, glucagon-like peptide-1, renal function, renal impairment, acute kidney injury, diabetic nephropathy, electrolytes, sodium, potassium, adverse effects. Randomised controlled trials, original papers, review articles and case reports are included in the present review. References of these articles were scrutinised for relevant articles.

## EXENATIDE

### **Effects of renal impairment on the metabolism of exenatide**

Exenatide is eliminated primarily *via* the kidneys<sup>[33]</sup>. Studies in pigs have shown that exenatide is cleared by glomerular filtration<sup>[34]</sup>.

In an open-label study, 31 subjects (one with T2DM) were given exenatide 5 or 10  $\mu$ g subcutaneously and divided in normal renal function group (Cockcroft-Gault creatinine clearance > 80 mL/min,  $n = 8$ ), mild renal impairment group (51-80 mL/min,  $n = 8$ ), moderate renal impairment group (31-50 mL/min,  $n = 7$ ) or end-stage renal disease (ESRD) requiring hemodialysis group ( $n = 8$ )<sup>[35]</sup>. Mean exenatide clearance was substantially reduced in subjects with ESRD (0.9 L/h) compared with the control group (3.4 L/h). Mean half-life of exenatide was 1.5 h in subjects with normal renal function, 2.1 h in patients with mild renal impairment, 3.2 h in patients with moderate renal impairment and 6 h in ESRD patients. The most common treatment-emergent adverse events were vomiting, nausea and headache. Although exenatide clearance was decreased by 13% in the mild renal impairment group compared with subjects with normal renal function, the tolerability of exenatide 10  $\mu$ g was acceptable and, consequently, this reduction in clearance did not seem clinically relevant. Exenatide clearance was decreased by 36% in patients with moderate renal impairment, but these patients also tolerated well both exenatide doses of 5 and 10  $\mu$ g. Patients with ESRD had significantly reduced clearance of exenatide by 84% and did not tolerate well the 5  $\mu$ g exenatide dose. Hence, no dosage adjustment of exenatide is required for patients with mild to moderate renal impairment, but the recommended starting dosage of 5  $\mu$ g exenatide may not be suitable for patients with ESRD or severe renal impairment (creatinine clearance < 30 mL/min)<sup>[35]</sup>.

Interestingly, a placebo-controlled, crossover study randomized elderly patients ( $\geq 75$  years,  $n = 15$ ) or controls ( $\geq 45$  to  $\leq 65$  years,  $n = 15$ ) with T2DM to single subcutaneous doses of exenatide 5  $\mu$ g, exenatide 10  $\mu$ g or placebo before a standardized breakfast over three consecutive days<sup>[36]</sup>. Although the dose-normalized plasma maximum concentration and exposure of exenatide were greater in elderly patients, no statistically significant between-age group differences were observed. These results imply that exenatide dose adjustments should be based on renal function rather than age in elderly T2DM patients<sup>[36]</sup> (Table 1).

### **Exenatide-induced acute kidney injury**

There are a number of case reports associating exenatide with the development of acute kidney injury<sup>[37-39]</sup>. The United States Food and Drug Administration (FDA) reported that between April 2005 and October 2008 there were 78 cases of altered kidney function (62 cases of acute renal failure and 16 cases of renal insufficiency) with exenatide<sup>[32]</sup>. Also, there was incomplete recovery of kidney function in many patients. Some of these cases

**Table 1 Studies of the effects of exenatide on renal function**

Ref.	Study details	Main findings
Li <i>et al</i> <sup>[57]</sup>	Administration of exendin-4 in human mesangial cells.	Exendin-4 decreased mRNA and protein levels of TGF-β1 and connective tissue growth factor. These effects were mainly dependent on the activation of adenylate cyclase <sup>[57]</sup> .
Carraro-Lacroix <i>et al</i> <sup>[70]</sup>	Investigation of the role of exendin-4 in modulating the activity of Na <sup>+</sup> /H <sup>+</sup> exchanger NHE3 in LLC-PK(1) cells.	GLP-1 receptor agonists modulate sodium homeostasis most likely by affecting NHE3 activity.
Liu <i>et al</i> <sup>[60]</sup>	<i>In vitro</i> administration of exendin-4 in spontaneously hypertensive rat renal arteries and aortic endothelial cells. Additionally, exendin-4 administration in renal arteries from hypertensive patients.	<i>In vitro</i> exendin-4 improved endothelium-dependent relaxation and restored renal blood flow in spontaneously hypertensive rat renal arteries and increased nitric oxide production in spontaneously hypertensive rat aortic endothelial cells <sup>[60]</sup> . <i>Ex vivo</i> exendin-4 administration improved endothelial function of renal arteries from hypertensive patients.
Park <i>et al</i> <sup>[58]</sup>	Administration of exendin-4 for 8 wk in a mouse model of diabetes (male <i>db/db</i> mice).	Exendin-4 improved intraperitoneal glucose tolerance test and decreased urinary albumin excretion in a dose-dependent manner. It also reduced glomerular hypertrophy, mesangial matrix expansion, TGF-β1 expression and type IV collagen accumulation, whereas it increased the renal immunoreactivity of peroxisome proliferator-activated receptor α and GLP-1 receptor-positive cells in the glomeruli of <i>db/db</i> mice.
Kodera <i>et al</i> <sup>[59]</sup>	Administration of exendin-4 in a streptozotocin-induced rat model of type 1 diabetes.	Exendin-4 improved albuminuria, glomerular hyperfiltration, glomerular hypertrophy and mesangial matrix expansion, reduced macrophage infiltration and protein levels of intercellular adhesion molecule-1 and type IV collagen and decreased oxidative stress and nuclear factor-κB activation in kidney tissue of the diabetic rats.
Mima <i>et al</i> <sup>[61]</sup>	Mice overexpressing protein kinase Cβ2 (results in a reduction of GLP-1 receptor expression) in endothelial cells (EC-PKCβ2Tg).	<i>In vivo</i> treatment with exendin-4 was partially effective to reduce glomerular pathology of both diabetic wild type and EC-PKCβ2Tg mice.
Hirata <i>et al</i> <sup>[62]</sup>	Exendin-4 for 12 wk in <i>db/db</i> mice (they have increased intra-renal angiotensin II concentration) and in angiotensin II-infused non-diabetic mice.	Exendin-4 inhibited the development of hypertension in <i>db/db</i> mice. Exendin-4 attenuated the delay of the urinary sodium excretion and elevation of blood pressure induced by a high-salt load. Exendin-4 prevented hypertension in angiotensin II-infused non-diabetic mice.
Liu <i>et al</i> <sup>[64]</sup>	A peptide analogue with exenatide (AC3174) was given for 4 wk via subcutaneous infusion in Dahl salt-sensitive rats.	The combination of AC3174 with captopril produced the most effective improvement in renal morphology (reduction of extensive sclerosis) in high salt diet rats compared with monotherapy. The combination of AC3174 with captopril also reduced the deleterious effects of high salt on posterior wall thickness and left ventricular mass.
Vaghasiya <i>et al</i> <sup>[66]</sup>	Exenatide treatment (14 d) in T2DM rats with induced renal ischemia for 30 min followed by reperfusion for 24 h.	Exenatide treatment normalized serum creatinine phosphokinase activity, liver function enzymes and antioxidant enzymes such as glutathione, superoxide dismutase, catalase and glutathione peroxidase (all <i>P</i> < 0.01).
Rieg <i>et al</i> <sup>[72]</sup>	Parenteral exendin-4 in wild-type mice and in mice lacking GLP-1 receptor. Additionally, administration of exendin-4 in diabetic <i>db/db</i> mice.	Parenteral exendin-4 in wild-type mice induced diuresis and natriuresis. These effects were associated with renal membrane expression of the Na <sup>+</sup> /H <sup>+</sup> NHE3, a site for cAMP-dependent protein kinase A. These effects were abolished in mice lacking the GLP-1 receptor. The administration of exendin-4 in diabetic <i>db/db</i> mice resulted in a reduction of renal fluid and Na <sup>+</sup> reabsorption.
Thomson <i>et al</i> <sup>[73]</sup>	Exenatide infusion in hydropenic male Wistar and Wistar-Froemter rats.	Exenatide infusion increased single-nephron glomerular filtration rate, early distal flow rate and urine flow rate and reduced proximal tubular reabsorption. These effects were observed without altering the efficiency of glomerulotubular balance, tubuloglomerular feedback responsiveness or the tonic influence of tubuloglomerular feedback.
Marina <i>et al</i> <sup>[78]</sup>	Exenatide administration in Wistar rats with normal serum concentration of glucose and potassium and in Wistar rats with hyperkalemia produced by intraperitoneal injection of 1.25% KCl solution.	Exenatide increased renal excretion of potassium in Wistar rats with normal serum concentration of glucose and potassium. Exenatide enhanced excretion of potassium in Wistar rats with hyperkalemia.
Simonsen <i>et al</i> <sup>[34]</sup>	Exendin-4 administration in anesthetised pigs ( <i>n</i> = 9).	Exenatide is solely cleared by glomerular filtration.
Linnebjerg <i>et al</i> <sup>[35]</sup>	Exenatide administration in 31 subjects (one with T2DM).	No dosage adjustment of exenatide is required for patients with mild to moderate renal impairment. In contrast, even the recommended starting dosage of 5 μg may not be suitable for patients with ESRD or severe renal impairment (creatinine clearance < 30 mL/min).
Linnebjerg <i>et al</i> <sup>[36]</sup>	Placebo-controlled, crossover study of elderly patients (≥ 75 yr, <i>n</i> = 15) or controls (≥ 45 to ≤ 65 yr, <i>n</i> = 15) with T2DM who received single subcutaneous doses of exenatide before a standardized breakfast.	Exenatide dose adjustments should be based on renal function rather than age in elderly T2DM patients.
Zhang <i>et al</i> <sup>[65]</sup>	31 patients with T2DM and microalbuminuria randomly received exenatide ( <i>n</i> = 13) or glimepiride ( <i>n</i> = 18) for 16 wk.	Similar reductions of fasting plasma glucose and HbA1c were observed between the two groups. Exenatide reduced body mass index (-5.95%), urinary type IV collagen and 24-h urinary albumin and urinary TGF-β1 (all <i>P</i> < 0.01).

Mendis <i>et al</i> <sup>[74]</sup>	Double-blind, randomized, crossover study of a single 10 µg subcutaneous injection of exenatide in healthy male volunteers ( <i>n</i> = 8).	Exenatide significantly increased after 2 h the urinary sodium/creatinine ratio compared with placebo ( <i>P</i> < 0.05). Exenatide administration was also associated with a significant increase of heart rate (+ 8.2 beats/min) and cardiac output, whereas a reduction in total peripheral resistance was observed (all <i>P</i> < 0.05). No change in blood pressure levels was observed.
US FDA <sup>[32]</sup>	Case reports of exenatide-induced acute kidney injury.	78 cases of altered kidney function (62 cases of acute renal failure and 16 cases of renal insufficiency) were reported with exenatide between April 2005 and October 2008.
Macconell <i>et al</i> <sup>[49]</sup>	Pooled analysis of 19 randomized, controlled trials of exenatide twice daily (5 µg and 10 µg) with 5594 intent-to-treat patients followed for 12-52 wk.	The incidence of renal impairment-related adverse events, including acute renal failure, was low (1.6 per 100 person-year for both groups) with no significant difference between groups (95%CI: -0.98-0.96). The most frequent adverse event with exenatide was transient, mild-to-moderate nausea (36.9% vs 8.3% in the pooled comparator).
Pendergrass <i>et al</i> <sup>[50]</sup>	Retrospective cohort of a large medical and pharmacy claims database including 491539 patients.	The adjusted risk for acute kidney injury among the patients with T2DM was not different between patients who received exenatide compared with patients who received other agents (hazard ratio = 0.77, 95%CI: 0.42-1.41, <i>P</i> = 0.40). Kaplan-Meier curves of time to acute kidney injury showed no significant differences between exenatide and other drugs.

TGF-β1: Transforming growth factor beta 1; GLP-1: Glucagon-like peptide 1; T2DM: Type 2 diabetes mellitus; ESRD: End-stage renal disease; FDA: Food and Drug Administration; NHE3: Exchanger isoform 3.

occurred in patients with pre-existing kidney disease. Ninety-five percent of the patients who experienced deterioration of kidney function had at least one risk factor for developing kidney problems, such as use of nephrotoxic medications, cardiac insufficiency or hypertension; these factors could have independently increased the risk for renal dysfunction<sup>[32]</sup>.

In most reports the acute kidney injury seems to be due to exenatide-induced prerenal acute failure. Main side effects of exenatide administration are nausea and vomiting, which result in decreased fluid intake and a significant loss of fluids<sup>[40]</sup>. The resulting volume contraction may lead to acute renal failure. Effects of GLP-1 such as natriuresis and a possible decrease in renal perfusion may also play a role in the loss of fluids and impairment of renal function<sup>[41,42]</sup>. The exenatide-induced volume contraction is mainly seen in patients who receive drugs that inhibit the renin-angiotensin system and aldosterone formation, an important homeostatic mechanism in states associated with volume depletion<sup>[43]</sup>. Furthermore, uremia per se is associated with nausea and may lead to a vicious circle of renal function deterioration<sup>[44]</sup>. In agreement with the above mechanisms, Weise *et al*<sup>[45]</sup> reported that four patients with nausea and vomiting experienced deterioration of kidney function following treatment with exenatide. There was incomplete recovery of kidney function in three patients. A kidney biopsy, which was performed in one patient, revealed ischemic glomeruli with moderate to severe interstitial fibrosis and early diabetic nephropathy<sup>[45]</sup>.

It should also be mentioned that other mechanisms of exenatide-induced acute kidney injury have been reported. For example, a 58-year-old man with poorly controlled T2DM was prescribed exenatide 5 µg twice daily as an alternative to treatment with insulin and experienced a deterioration in his kidney function<sup>[46]</sup>. Treatment with exenatide was initially associated with significant loss of weight (from 83 kg to 77 kg). However,

after 2 mo an increase in serum creatinine concentration [from 1.36 mg/dL (120 µmol/L) to 1.91 mg/dL (169 µmol/L)] was observed, despite the fact that the patient was systemically well, euvolemic and normotensive. He did not take any non-steroidal anti-inflammatory drugs or other non-prescribed medications. One month later, the serum creatinine concentration had increased to 2.36 mg/dL (209 µmol/L) and exenatide was stopped. There was further deterioration in his kidney function over the next month [(creatinine concentration 4.19 mg/dL (370 µmol/L)] and treatment with indapamide, candesartan and amlodipine was stopped. His urine contained red and white blood cells. An ultrasound examination revealed two normal kidneys. There was no evidence of a skin rash and the full blood count was normal. A kidney biopsy revealed active, moderately severe diffuse tubulointerstitial nephritis. The inflammatory infiltrate included many eosinophils, implying a drug-induced reaction. There was active tubular damage with desquamation of epithelium. He was treated with prednisolone 50 mg daily and an improvement in kidney function was observed within a few days. Prednisolone was gradually reduced over the next few weeks to a daily dose of 10 mg with a further improvement in kidney function [creatinine concentration 1.98 mg/dL (175 µmol/L)]<sup>[46]</sup>.

Similarly, in 2010, Bhatti *et al*<sup>[47]</sup> reported that two patients had experienced deterioration in kidney function following treatment with exenatide. One patient had no clinical evidence of dehydration and no response to rehydration was seen. This patient had hematuria and proteinuria and interstitial nephritis was suspected; however, a kidney biopsy was not performed. The patient was treated with prednisolone and there was incomplete recovery of kidney function. The other patient had clinical evidence of dehydration and there was improvement in kidney function following rehydration<sup>[47]</sup>.

Based on the above evidence FDA proposed that exenatide should not be used in patients with severe re-

**Table 2 Studies of the effects of liraglutide on renal function**

Author	Study details	Main findings
Kim <i>et al</i> <sup>[89]</sup>	Liraglutide administration in <i>Glp1r(-/-)</i> , <i>Nppa(-/-)</i> or wild type mice.	Liraglutide led to relaxation of aortic rings through a GLP-1 receptor-dependent but endothelium-independent manner. Liraglutide did not induce ANP secretion and did not result in vasorelaxation or blood pressure reduction in <i>Glp1r(-/-)</i> or <i>Nppa(-/-)</i> mice. Refeeding was associated with an increase in ANP levels in wild-type mice, whereas this effect was not observed in <i>Glp1r(-/-)</i> mice. Liraglutide administration led to increase of urine sodium excretion in wild-type, whereas this effect was abolished in <i>Nppa(-/-)</i> mice. These findings suggest a gut-heart axis, which is both GLP-1 receptor-dependent and ANP-dependent and regulates blood pressure.
Hendarto <i>et al</i> <sup>[90]</sup>	Liraglutide administration in streptozotocin-induced type 1 diabetes rats. Additionally, incubation of cultured renal mesangial cells with liraglutide for 48 h.	Liraglutide administration in streptozotocin-induced diabetic rats normalized the increased urinary albumin excretion and oxidative stress markers, as well as the expression of NADPH oxidase components, TGF-β1 and fibronectin in renal tissues. The incubation of cultured renal mesangial cells with liraglutide inhibited NADPH-dependent superoxide production in a dose-dependent manner, an effect that was abolished by a protein kinase A inhibitor and an adenylate cyclase inhibitor.
Malm-Erfjelt <i>et al</i> <sup>[82]</sup>	Administration of radio-labelled liraglutide in seven healthy males.	Liraglutide is metabolized by DPP-IV similarly with the native GLP-1, but at a much slower rate. No intact liraglutide was excreted in urine and feces.
Jacobsen <i>et al</i> <sup>[83]</sup>	A single dose of liraglutide 0.75 mg was given subcutaneously in 30 subjects (24 with varying degrees of renal impairment and 6 with normal renal function).	No significant effect of reduced creatinine clearance on the pharmacokinetics of liraglutide was observed. No association was found between the degree of renal impairment and the risk of adverse events.
Davidson <i>et al</i> <sup>[84]</sup>	A meta-analysis of the 6 LEAD (Liraglutide Effect and Action in Diabetes) studies which analysed data from patients with T2DM administered once-daily liraglutide (1.2 or 1.8 mg) or placebo as either monotherapy or in combination with oral antidiabetic drugs for 26 wk.	Mild renal impairment (determined by the Cockcroft-Gault equation) had no significant effect on the efficacy and safety of liraglutide. No significant differences in the rates of nausea, renal injury or minor hypoglycemia were observed between liraglutide and placebo in patients with mild renal impairment. No significant effect of mild renal impairment on HbA1c reduction was observed. However, a trend towards increased nausea was observed with liraglutide in the small number of patients with moderate or severe renal impairment.

GLP-1: Glucagon-like peptide 1; ANP: Atrial natriuretic peptide; TGF-β1: Transforming growth factor beta 1; DPP-IV: Dipeptidyl peptidase IV; T2DM: Type 2 diabetes mellitus; HbA1c: Glycated haemoglobin.

renal impairment (creatinine clearance < 30 mL/min) or ESRD and caution should be applied when initiating or increasing doses of exenatide from 5 to 10 µg in patients with moderate renal impairment (creatinine clearance 30-50 mL/min)<sup>[32]</sup>. Furthermore, the once weekly exenatide, which was recently approved by the FDA, is not recommended in patients with severe renal impairment or ESRD and caution is warranted in patients with renal transplantation or moderate renal impairment<sup>[48]</sup>.

However, it should be mentioned that the reported cases of altered renal function with exenatide represent a small percentage of the total number of patients who have used the drug (more than 6.6 million prescriptions)<sup>[32]</sup>. Furthermore, recent analyses do not associate exenatide use and acute kidney injury. In a pooled analysis of 19 completed, randomized, controlled clinical trials of exenatide twice daily (5 or 10 µg) 5594 intent-to-treat patients who were followed for 12-52 wk were included<sup>[49]</sup>. Transient, mild-to-moderate nausea was the most frequent adverse event with exenatide (36.9% vs 8.3% in the pooled comparator). Renal impairment-related adverse events, including acute renal failure, were low (1.6 per 100 person-years for both groups) and no significant difference was observed between groups (95%CI: -0.98 to 0.96)<sup>[49]</sup>. Additionally, a retrospective cohort study of a large medical and pharmacy claims database including data for 491539 patients was recently published<sup>[50]</sup>. The unadjusted incidence rates of acute kidney injury were higher in patients with T2DM (1.13 cases/100 patient-

years) compared with the subjects without T2DM (0.34 cases/100 patient-years). The unadjusted incidence rates of acute kidney injury were similar between exenatide users (0.94 cases/100 patient-years) and the other T2DM patients (1.02 cases/100 patient-years). Moreover, the adjusted risk of acute kidney injury did not differ between patients who received exenatide and T2DM patients who received other agents (HR = 0.77, 95%CI: 0.42-1.41, P = 0.40). Similar results were observed when analysis was restricted to the patients with at least one risk factor for acute kidney injury [(exenatide user: HR = 0.52, 95%CI: 0.45-1.50), P = 0.40]. Finally, when Kaplan-Meier curves of time to acute kidney injury were used, no significant differences between the groups receiving exenatide or other drugs were observed<sup>[50]</sup> (Table 1).

**Effects of exenatide on renal function**

**Effects on diabetic nephropathy:** Diabetic nephropathy is histologically characterized by the accumulation of extracellular matrix proteins in the glomerular mesangium<sup>[51]</sup>. It has been shown that these processes are mediated by the transforming growth factor-beta 1 (TGF-β1), which is expressed in renal tissues of patients with diabetic nephropathy<sup>[52-54]</sup>. The TGF-β1 is a major fibrogenic growth factor in the pathogenesis of glomerulosclerosis and interstitial fibrosis, since it induces collagen and matrix synthesis and the expression of connective tissue growth factor, mRNA and proteins<sup>[55]</sup>. Hence, TGF-β1 is a useful marker of the fibrotic response<sup>[56]</sup>.

Treatment with exendin-4 improves the renal interstitial fibrosis in culture and animal models of diabetic nephropathy. The administration of exendin-4 in human mesangial cells decreased the mRNA and protein levels of TGF- $\beta$ 1 and connective tissue growth factor, effects that were mainly dependent on the activation of adenylate cyclase<sup>[57]</sup>. In a mouse model of diabetes (male *db/db* mice) the administration of 1 nmol/kg exendin-4 for 8 wk resulted in improvement of intraperitoneal glucose tolerance test compared with the control group ( $P < 0.05$ )<sup>[58]</sup>. Fasting blood glucose, glycated hemoglobin (HbA1c) and creatinine concentrations did not significantly differ among *db/db* mice, whereas urinary albumin excretion was significantly decreased in a dose-dependent manner with exendin-4 compared with control *db/db* mice ( $P < 0.005$ ). Renal histology studies showed that treatment with exendin-4 resulted in a significant reduction of glomerular hypertrophy, mesangial matrix expansion, TGF- $\beta$ 1 expression, type IV collagen accumulation and associated glomerular lipid accumulation. Furthermore, fewer infiltrating inflammatory and apoptotic cells together with an increase in the renal immunoreactivity of peroxisome proliferator-activated receptor  $\alpha$  and GLP-1 receptor-positive cells were observed in the glomeruli of *db/db* mice treated with exendin-4 compared with control group<sup>[58]</sup>. The administration of exendin-4 (10  $\mu$ g/kg per day) in a streptozotocin-induced rat model of type 1 diabetes did not significantly alter blood pressure or body weight, but resulted in improvement of albuminuria, glomerular hyperfiltration, glomerular hypertrophy and mesangial matrix expansion. A reduction in protein levels of intercellular adhesion molecule-1 and type IV collagen together with a decrease in macrophage infiltration, oxidative stress and nuclear factor- $\kappa$ B activation were also observed in the kidney tissue of diabetic rats<sup>[59]</sup>.

Furthermore, there are other possible mechanisms that GLP-1 receptor agonists improve diabetic nephropathy. Exendin-4 *in vitro* improved endothelium-dependent relaxation and restored renal blood flow in spontaneously hypertensive rat renal arteries and increased nitric oxide production in spontaneously hypertensive rat aortic endothelial cells<sup>[60]</sup>. Furthermore, *ex vivo* exendin-4 administration improved endothelial function of renal arteries from hypertensive patients. It seems that GLP-1 receptor agonists improve endothelial function by restoring nitric oxide bioavailability<sup>[60]</sup>.

Other authors have shown that the protective action of GLP-1 in glomerular endothelial cells is partly mediated *via* its own receptor by the activation of protein kinase A<sup>[61]</sup>. It was also proposed that the presence of T2DM induces the activation of protein kinase C $\beta$  isoform, which results in a reduction of GLP-1 receptor expression and an increase of its degradation through ubiquitination and/or enhancement of angiotensin II-mediated mechanisms<sup>[61]</sup>. Specifically, mice overexpressing protein kinase C $\beta$ 2 in endothelial cells (EC-PKC $\beta$ 2Tg) had decreased GLP-1 receptor expression

and enhanced angiotensin II-mediated effects. Although diabetes and hyperglycemia blunted *via* PKC $\beta$  activation the protective actions of GLP-1, treatment with exendin-4 *in vivo* was still partially effective to reduce glomerular pathology of both diabetic wild type and EC-PKC $\beta$ 2Tg mice<sup>[61]</sup>. In this context, exendin-4 has been described to exert anti-hypertensive effects through the attenuation of angiotensin II-mediated effects. A study showed that treatment with exendin-4 for 12 wk inhibited the development of hypertension in *db/db* mice with increased intra-renal angiotensin II concentration<sup>[62]</sup>. Furthermore, exendin-4 attenuated the delay of urinary sodium excretion and the elevation of blood pressure induced by a high-salt load in *db/db* mice. Exendin-4 also prevented angiotensin II-induced hypertension in angiotensin II-infused non-diabetic mice<sup>[62]</sup>. Of note, a 2-h infusion of GLP-1 in 12 healthy young males was associated with a significant reduction of angiotensin II levels with no parallel change in the concentration of renin and aldosterone or the urinary excretion of angiotensinogen<sup>[63]</sup>.

There is also evidence of a beneficial role of the combination of GLP-1 receptor agonists with angiotensin converting enzyme inhibitors. A peptide analogue with exenatide (AC3174 1.7 pmol/kg per minute) was given for 4 wk *via* subcutaneous infusion in Dahl salt-sensitive (DSS) rats<sup>[64]</sup>. The administration of AC3174, captopril or AC3174 plus captopril improved renal function ( $P < 0.05$ ), but the combination of AC3174 with captopril produced the most effective improvement in renal morphology (reduction of extensive sclerosis) in these high salt diet rats. The combination of AC3174 with captopril also reduced the deleterious effects of high salt on posterior wall thickness and left ventricular mass ( $P < 0.05$ ). It should be mentioned that the administration of GLP-1 did not result in improvement of cardiovascular parameters and survival, implying that GLP-1 receptor agonists are more potent peptides or have at least partly different mechanism of action<sup>[64]</sup>.

The effects of exenatide on diabetic nephropathy were examined in 31 patients with T2DM and microalbuminuria, who randomly received exenatide ( $n = 13$ ) or glimepiride treatment ( $n = 18$ ) for 16 wk<sup>[65]</sup>. Exenatide resulted in a significant reduction of body mass index (BMI) by 5.95% (from 24.9 to 23.3 kg/m<sup>2</sup>), whereas glimepiride treatment did not significantly alter BMI levels (-0.25%, from 24.8 to 24.7 kg/m<sup>2</sup>). Similar reductions of fasting plasma glucose and HbA1c were observed between the two groups. Exenatide resulted in a significant reduction of 24 h urinary albumin and urinary TGF- $\beta$ 1 (all  $P < 0.01$ ), whereas these variables did not significantly change with glimepiride. Additionally, the excretion of urinary type IV collagen was significantly decreased with exenatide (-25.3%) compared with glimepiride (-1.6%,  $P < 0.005$ )<sup>[65]</sup>.

Interestingly, a study showed that exenatide exerts protective effects on liver injury induced by renal ischemia reperfusion in diabetes<sup>[66]</sup>. Specifically, a previous treat-

ment with exenatide for 14 d in T2DM rats with induced renal ischemia for 30 min followed by reperfusion for 24 h significantly normalized serum creatinine phosphokinase activity, liver function enzymes and antioxidant enzymes such as glutathione, superoxide dismutase, catalase and glutathione peroxidase (all  $P < 0.01$ )<sup>[66]</sup>.

Overall, GLP-1 receptor agonists seem to improve the histologic changes and markers of diabetic nephropathy. These effects seem promising for the treatment of T2DM patients. However, it should be mentioned that most of the evidence is based on animal studies and the extrapolation of these observations to human physiology should be done with caution.

**Effects on water and electrolyte balance:** Excreted sodium is re-absorbed by 60%-70% in the proximal nephron, mainly by the  $\text{Na}^+/\text{H}^+$  exchanger isoform 3 (NHE3)<sup>[67]</sup>. GLP-1 receptors are expressed in the proximal tubule<sup>[68]</sup>. There is evidence from animal studies that GLP-1 modulates sodium homeostasis in the kidney *via* the GLP-1 receptor in proximal tubular cells. Specifically, GLP-1 administration in porcine proximal tubular kidney cells led to an inhibition of sodium re-absorption after 3 h of incubation. In contrast, the use of a DPP-IV inhibitor in combination with exendin-4 or GLP-1 did not alter significantly glucose and sodium uptake and transport<sup>[68]</sup>.

It was also demonstrated that GLP-1 can stimulate renal excretion of sodium in rats and humans, most likely by affecting NHE3 activity<sup>[41,69,70]</sup>. The administration of GLP-1 in rats (1  $\mu\text{g}/\text{kg}$  per minute intravenously for 60 min) increased urine flow, fractional excretion of sodium, potassium and bicarbonate and was accompanied by increases in renal plasma flow and glomerular filtration rate (GFR)<sup>[71]</sup>. GLP-1 receptor-mRNA expression was restricted to glomerulus and proximal convoluted tubule. It was also shown that GLP-1 significantly reduced NHE3-mediated bicarbonate reabsorption in rat renal proximal tubule, through a protein kinase A-dependent mechanism<sup>[71]</sup>.

Another study reported that parenteral administration of exendin-4 in wild-type mice induced diuresis and natriuresis<sup>[72]</sup>. These effects were associated with increases in glomerular filtration rate, fractional urinary fluid and  $\text{Na}^+$  excretion. Furthermore, these effects were associated with renal membrane expression of the NHE3, a site for cAMP-dependent protein kinase A. These effects were abolished in mice lacking the GLP-1 receptor and were independent of adenylate cyclase 6. Of interest, the administration of parenteral DPP-IV inhibitor alogliptin in these wild-type mice induced diuresis and natriuresis, which were independent of the presence of the GLP-1 receptor or alterations in the phosphorylated NHE3. These results may imply mechanistic differences between exendin-4 and DPP-IV inhibition in the induction of diuresis and natriuresis under normal states. Notably, the administration of exendin-4 in diabetic *db/db* mice resulted in a reduction of renal fluid and  $\text{Na}^+$

reabsorption, whereas these effects were not observed when diabetic *db/db* mice were given alogliptin. These results imply significant differences between exendin-4 and DPP-IV inhibition in a T2DM mice model, since GLP-1 receptor-mediated natriuretic mechanisms were preserved, whereas DPP-IV inhibitor-dependent mechanisms were abolished<sup>[72]</sup>.

A recent study described a role of exenatide as a proximal diuretic and renal vasodilator<sup>[73]</sup>. Exenatide infusion (1 nmol/h *iv*) in hydropenic male Wistar and Wistar-Froemter rats increased single-nephron glomerular filtration rate by 33%-50%, reduced proximal tubular reabsorption by 20%-40%, doubled early distal flow rate and increased urine flow rate six-fold without altering the efficiency of glomerulotubular balance, tubuloglomerular feedback responsiveness or the tonic influence of tubuloglomerular feedback<sup>[73]</sup>.

A recent randomized, double-blinded, single-day, crossover trial showed that the infusion of GLP-1 for 2 h in 12 healthy young males increased renal sodium clearance by 40% ( $P = 0.007$ ) and decreased angiotensin II levels by 19% ( $P = 0.003$ ), whereas no change in renin, aldosterone or the urinary excretion of angiotensinogen was observed<sup>[63]</sup>. The infusion of GLP-1 did not significantly alter the GFR (assessed with <sup>51</sup>Cr-EDTA), renal plasma flow (assessed with <sup>125</sup>I-hippuran) or blood pressure levels, but induced a small transient increase in heart rate<sup>[63]</sup>. In another study the urinary sodium/creatinine ratio was significantly increased (12.4 mmol/mmol, 95%CI: 4.6-20.2,  $P < 0.05$ ) compared with placebo 2 h after a single 10  $\mu\text{g}$  subcutaneous injection of exenatide in eight healthy male volunteers<sup>[74]</sup>. Furthermore, exenatide administration was associated with a significant increase of heart rate (8.2 beats/min, 95%CI: 4.2-12.2,  $P < 0.01$ ) and cardiac output (1.2 L/min, 95%CI: 0.42-20.3,  $P < 0.05$ ), whereas a reduction in total peripheral resistance ( $P < 0.05$ ) was observed. These effects were not linked with any change in blood pressure levels<sup>[74]</sup>. Therefore, exenatide has both vasodilator and natriuretic properties. Although the effects of short-time administration of exenatide were not associated with significant changes in blood pressure levels, they may be related with the reduction in blood pressure that was observed in clinical studies examining the use of GLP-1 receptor agonists in patients with T2DM<sup>[75,76]</sup>.

Furthermore, a possible role of exenatide in the human osmoregulation system has been proposed. A study (article in Russian, so no more details than the abstract could be used) showed that water load of 0.7% of body weight caused significant increase in urine excretion in 55 subjects (38 patients with T2DM)<sup>[77]</sup>, but the rise of diuresis was depended on the increase in solute-free water clearance when exenatide 10  $\mu\text{g}$  was administered with the water load<sup>[77]</sup>.

Finally, there is evidence of a possible role of exenatide in the normalization of potassium balance *via* renal mechanisms. The administration of exenatide (0.015-0.5 nmol/100 g body weight) to Wistar rats with

normal serum concentration of glucose and potassium increased renal excretion of potassium from  $7 \pm 1$  to  $16 \pm 1 \mu\text{mol/h}$  per 100 g body weight ( $P < 0.05$ )<sup>[78]</sup>. Moreover, exenatide enhanced excretion of potassium in Wistar rats with hyperkalemia produced by intraperitoneal injection of 1.25% KCl solution; specifically, during the first post-injection hour, potassium excretion was increased from  $47 \pm 9 \mu\text{mol/h}$  per 100 g body weight with potassium load alone to  $97 \pm 11 \mu\text{mol/h}$  per 100 g body weight with exenatide ( $P < 0.05$ )<sup>[78]</sup> (Table 1).

## LIRAGLUTIDE

### **Effects of renal impairment on liraglutide metabolism**

Liraglutide shares a 97% structural homology with human GLP-1, has a longer half-life than the native hormone and undergoes a generalized proteolysis without elimination *via* the kidneys<sup>[33,79-81]</sup>. A study in seven healthy males who received radio-labelled liraglutide showed that liraglutide is metabolized by DPP-IV and neutral endopeptidase, similarly with the native GLP-1 but at a much slower rate. Furthermore, the results of this study showed that liraglutide is mainly degraded within the body since no intact liraglutide was excreted in urine and feces<sup>[82]</sup>.

Renal impairment does not alter significantly the pharmacokinetic profile of liraglutide<sup>[33]</sup>. In a study a single dose of liraglutide 0.75 mg subcutaneously was given in 30 subjects, 24 with varying degrees of renal impairment and six with normal renal function<sup>[83]</sup>. The regression analysis of log [area under the curve (AUC)] of liraglutide for subjects with normal renal function and mild-to-severe renal impairment did not show any significant effect of reduced creatinine clearance on the pharmacokinetics of liraglutide. Furthermore, the AUC ratio of the subject with the lowest and the subject with the highest creatinine clearance was not significant (0.88, 95%CI: 0.58-1.34,  $P > 0.05$ ). It should be mentioned that the between-group comparisons of the AUC of liraglutide did not show equivalence, since the estimated ratio of AUC(severe)/AUC(healthy) was 0.73 (90% 0.57-0.94) and the ratio of AUC(continuous ambulatory peritoneal dialysis)/AUC(healthy) was 0.74 (90%CI: 0.56-0.97). However, no association was found between the degree of renal impairment and the risk of adverse events<sup>[83]</sup>. Based on this study, liraglutide can be used safely in patients with varying degrees of renal impairment.

A meta-analysis of the 6 LEAD (Liraglutide Effect and Action in Diabetes) studies analysed data from patients with T2DM administered once-daily liraglutide (1.2 or 1.8 mg) or placebo as either monotherapy or in combination with oral antidiabetic drugs for 26 wk. The patients were grouped as having normal renal function (Cockcroft-Gault creatinine clearance  $> 89 \text{ mL/min}$ ), mild renal impairment ( $60 \text{ mL/min} \leq \text{creatinine clearance} \leq 89 \text{ mL/min}$ ) and moderate or severe renal impairment (creatinine clearance  $< 60 \text{ mL/min}$ )<sup>[84]</sup>. Liraglutide administration was well tolerated in patients with

mild renal impairment since no significant differences in the rates of nausea, renal injury or minor hypoglycemia were observed compared with placebo. No significant effect of mild renal impairment on HbA1c reduction was observed. However, a trend towards increased nausea was observed with liraglutide in the small number of patients with moderate or severe renal impairment. Overall, this meta-analysis showed that mild renal impairment (determined by the Cockcroft-Gault equation) did not have a significant effect on the efficacy and safety of liraglutide<sup>[84]</sup>.

However, the long-term data regarding the use of liraglutide in patients with moderate-to-severe renal impairment is limited. Hence, the summary of product characteristics of liraglutide proposes no dose adjustment for patients with mild renal impairment (creatinine clearance 60-90 mL/min), but does not recommend the use of the drug in patients with moderate and severe renal impairment including patients with ESRD<sup>[85]</sup>.

A randomised, placebo-controlled, double-blinded trial aiming to test safety and efficacy of treatment with liraglutide in patients with T2DM and dialysis-dependent ESRD was recently announced<sup>[86]</sup>. In this trial 20 patients with T2DM and ESRD and 20 matched patients with T2DM and normal kidney function will receive liraglutide for 12 wk (9 visits) in an individually titrated dose of 0.6, 1.2 or 1.8 mg/d or placebo. The primary endpoint is dose-corrected plasma trough liraglutide concentration at the final trial visit aiming to determine potential accumulation in the ESRD group. Glycemic control,  $\beta$ -cell response, cardiovascular parameters, various biomarkers and adverse events will also be assessed<sup>[86]</sup> (Table 2).

### **Liraglutide-induced acute kidney injury**

A case report described a 53-year-old Caucasian woman who had started 1 mo earlier subcutaneous liraglutide 1.8 mg/d for uncontrolled T2DM and was admitted with serum creatinine concentration of 22.8 mg/dL and blood urea nitrogen of 150 mg/dL<sup>[87]</sup>. She had lost 8.9 kg in the previous month after severe and progressively worsening gastrointestinal symptoms leading to dehydration. Other potential causes of renal failure and adverse drug reactions due to other drugs such as ciprofloxacin and quinapril were ruled out by laboratory investigation and renal biopsy. Renal biopsy showed that liraglutide was a likely cause of acute kidney injury through the development of acute tubular necrosis. The patient was treated with discontinuation of liraglutide, volume repletion, and hemodialysis<sup>[87]</sup>.

Another case report described a 56-year-old man with T2DM who started liraglutide aiming to a gradual reduction of insulin because of hypoglycemic episodes<sup>[88]</sup>. Three months later the patient reported that his early morning glucose levels were elevated and he had nocturia. Laboratory results revealed an increase in his creatinine concentration [from 1.1 mg/dL (101  $\mu\text{mol/L}$ ) to 1.56 (138  $\mu\text{mol/L}$ )]. Liraglutide, ramipril, indapamide and metformin were stopped and insulin was restarted.

His renal function was completely recovered a few weeks later. Similarly, a 65-year-old man receiving liraglutide had polyuria and polydipsia. His HbA1c was 12.9% and his urine dipstick showed glycosuria. Laboratory results revealed an increase in his serum creatinine [1.8 mg/dL (159  $\mu$ mol/L)]. Liraglutide was discontinued and insulin was initiated. Serum creatinine levels returned to normal [1.2 mg/dL (109  $\mu$ mol/L)] after 5 wk of withholding liraglutide. After 2 mo of initiation of insulin his HbA1c dropped by 1.9%. The most likely mechanism for the renal impairment in these patients is volume depletion causing renal impairment<sup>[88]</sup>.

Overall, despite these case reports, liraglutide seems to be a safe drug in terms of kidney function. However, clinicians should be cautious in patients receiving liraglutide and have uncontrolled T2DM with polyuria and polydipsia or have symptoms that predispose to volume depletion (for example vomiting) (Table 2).

### Effects of liraglutide on renal function

A recent study in mice showed that GLP-1 receptors are localized and expressed in cardiac atria<sup>[89]</sup>. Furthermore, it was shown that GLP-1 receptor activation is associated with the secretion of atrial natriuretic peptide (ANP) and a reduction of blood pressure. Specifically, liraglutide did not induce ANP secretion and did not result in vasorelaxation or blood pressure reduction in *Glp1r(-/-)* or *Nppa(-/-)* mice. Moreover, refeeding was associated with an increase in ANP levels in wild-type mice, whereas this effect was not observed in *Glp1r(-/-)* mice. On the other hand, liraglutide administration increased urine sodium excretion in wild-type mice, whereas this effect was abolished in *Nppa(-/-)* mice. These findings suggest a gut-heart axis that regulates blood pressure, which is both GLP-1 receptor-dependent and ANP-dependent. Furthermore, it was shown that liraglutide led to relaxation of aortic rings through a GLP-1 receptor-dependent, indirect ANP-dependent and endothelium-independent manner, since a conditioned medium from liraglutide-treated hearts resulted in relaxation of aortic rings but did not directly increase the amount of cyclic guanosine monophosphate (cGMP, associated with the function of ANP) or relax pre-constricted aortic rings. There is evidence that the Rap guanine nucleotide exchange factor Epac2 (also known as Rapgef4, an exchange protein activated by cAMP) may mediate the association of GLP-1 receptor activation and ANP secretion, since cardiomyocyte GLP-1 receptor activation induced the translocation of the Epac2 to the membrane, whereas Epac2 deficiency did not induce ANP secretion through GLP-1 receptor stimulation<sup>[89]</sup>.

A study showed that liraglutide inhibits oxidative stress and albuminuria in streptozotocin-induced type 1 diabetes mellitus rats through a protein kinase A-mediated inhibition of renal NADPH oxidases<sup>[90]</sup>. Specifically, diabetic rats were randomly treated with liraglutide (0.3 mg/kg 12 h subcutaneously) for 4 wk. The administration of liraglutide normalized the increased urinary albu-

min excretion and oxidative stress markers, as well as the expression of NADPH oxidase components, TGF- $\beta$ 1 and fibronectin in renal tissues, without affecting plasma glucose levels or body weight of streptozotocin-induced diabetic rats. Additionally, the authors conducted *in vitro* experiments which showed that incubation of cultured renal mesangial cells with liraglutide for 48 h inhibited NADPH-dependent superoxide production in a dose-dependent manner, an effect that was abolished by a protein kinase A inhibitor and an adenylate cyclase inhibitor<sup>[90]</sup>.

Another study evaluated the effects of liraglutide on tumour necrosis factor- $\alpha$ -induced injury of the human umbilical vein endothelial cells and showed that the drug inhibits protein kinase A, NADPH oxidase and nuclear factor- $\kappa$ B signaling and upregulates protective antioxidative enzymes. Consequently, liraglutide exerts significant anti-oxidative and anti-inflammatory effects on endothelial cells<sup>[91]</sup>. If these protective effects are also evident in renal cells remains to be established (Table 2).

## CONCLUSION

Chronic kidney disease is a common complication of T2DM resulting in a progressive deterioration of renal function<sup>[92]</sup>. However, many of the antidiabetic drugs are contraindicated or require dosage adjustments in patients with renal impairment. Based on the current evidence, exenatide should not be given in patients with severe renal impairment or ESRD because the drug is eliminated by renal mechanisms. Liraglutide, although it is not eliminated by renal or hepatic mechanisms, should be used with caution since there are only limited data in patients with renal or hepatic impairment<sup>[80,81,93]</sup>.

Furthermore, current evidence shows that these drugs exert protective role in diabetic nephropathy with mechanisms that many times are independent of their glucose-lowering effect. GLP-1 receptor agonists have also been shown to influence water and electrolyte balance. Although most of these effects have been demonstrated in culture or animal models and their mechanism of action need to be better elucidated, they may represent new ways to improve or even prevent diabetic nephropathy. It should be mentioned that animal studies should be interpreted with caution, since a number of drugs evaluated in rodents with induced diabetes were ineffective in clinical trials (for example the advanced glycation endproduct inhibitors).

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## Effect of ethnicity on weight loss among adolescents 1 year after bariatric surgery

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weight outcomes vary by ethnicity in a large, nationally representative sample of adolescents.

**METHODS:** The Bariatric Outcomes Longitudinal Database was used for analysis and contains data on surgeries performed on adolescents from 2004 to 2010 from 423 surgeons at 360 facilities across the United States Adolescents ( $n = 827$ ) between 11 and 19 years old who underwent either gastric bypass or adjustable gastric banding surgery were included in the analysis. Outcome measures included changes in anthropometric measurements [weight (kg) and body mass index] from baseline to 3 ( $n = 739$ ), 6 ( $n = 512$ ), and 12 ( $n = 247$ ) mo after surgery.

**RESULTS:** A year after patients underwent either gastric bypass (51%) or adjustable gastric banding (49%) surgery, mean estimated weight loss for all ethnic groups differed by a maximum of only 1.5 kg, being 34.3 kg (95%CI: 30.0-38.5 kg) for Hispanics, 33.8 kg (95%CI: 27.3-40.3 kg) for non-Hispanic blacks, and 32.8 kg (95%CI: 30.9-34.7 kg) for non-Hispanic whites. No overall pairwise group comparisons were significant, indicating that no ethnic group had better weight loss outcomes than did another.

**CONCLUSION:** Bariatric surgery substantially reduces the weight of severely obese adolescents at 1 year post-procedure with little variation by ethnicity and/or gender. These results suggest that bariatric surgery is a safe and reasonable treatment for all severely obese adolescents with the appropriate indications.

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**Key words:** Bariatric surgery; Gastric bypass surgery; Adjustable gastric band surgery; Adolescents; Severe obesity; Ethnicity; Weight loss

**Core tip:** Bariatric surgery is one of the few effective

### Abstract

**AIM:** To investigate whether or not bariatric surgery

treatments for severe obesity. Among adults, outcomes of bariatric surgery differ by ethnicity. We tested whether this relationship is also true among adolescents. Outcome measures included changes in anthropometric measurements (weight and body mass index) from baseline ( $n = 827$ ) to 1 year after surgery. Our results support the conclusion that bariatric surgery can substantially reduce weight in severely obese adolescents for at least 1 year, irrespective of their race or ethnicity. Ethnicity is a reasonable and safe treatment for all severely obese adolescents with the appropriate indications.

Messiah SE, Lopez-Mitnik G, Winegar D, Sherif B, Arheart KL, Reichard KW, Michalsky MP, Lipshultz SE, Miller TL, Livingstone AS, de la Cruz-Muñoz N. Effect of ethnicity on weight loss among adolescents 1 year after bariatric surgery. *World J Diabetes* 2013; 4(5): 202-209 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i5/202.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i5.202>

## INTRODUCTION

The Centers for Disease Control and Prevention (CDC) recently reported that the prevalence of childhood obesity in the United States has stabilized over the past few years, although it remains high among all age, sex, and ethnic groups<sup>[1]</sup>. An estimated 12% of all United States children between 2 and 19 years old have a body mass index (BMI)  $\geq$  97<sup>th</sup> percentile for age and sex. Moreover, the prevalence of obesity differs significantly among racial and ethnic groups. In 2009-2010, 21% of Hispanic and 24% of non-Hispanic black children and adolescents were obese, whereas 14% of non-Hispanic white children and adolescents were obese.

Childhood obesity has several health-related consequences that, until recently, were documented only in adulthood, including hypertension, insulin resistance, glucose intolerance, and dyslipidemia<sup>[2]</sup>. In turn, these conditions are risk factors for type 2 diabetes and cardiovascular disease in both childhood and adulthood<sup>[3-5]</sup>. Weight-loss surgery among both adults and adolescents has become increasingly recognized as effective treatment for severe obesity and several of its related comorbidities<sup>[6-10]</sup>. Current studies suggest that neither pharmacologic nor dietary treatment can maintain weight loss in obese adolescents as effectively as can weight-loss surgery<sup>[11,12]</sup>.

Several studies have shown that among adults, weight loss outcomes after bariatric surgery differ by ethnicity with some studies reporting better outcomes among non-Hispanic whites than among non-Hispanic blacks<sup>[13-16]</sup>. Although the literature on outcomes of bariatric surgery in adolescents has increased exponentially, many studies are small and usually from single institutional series<sup>[8]</sup>. As a result, patients are not geographically or ethnically diverse, despite the fact that obesity disproportionately affects ethnic minorities<sup>[1]</sup>.

Accordingly, we analyzed data from the Bariatric Outcomes Longitudinal Database (BOLD), a large national database that tracks outcomes in patients from a wide geographical area who have undergone bariatric surgery. We report here the results of our analysis, which indicate that weight loss in a large cohort of severely obese adolescents 1 year after bariatric surgery did not differ by ethnicity.

## MATERIALS AND METHODS

### BOLD database

Data for the BOLD database are collected prospectively from participants in the Bariatric Surgery Center of Excellence (BSCO) program sponsored by the American Society for Metabolic and Bariatric Surgery (ASMBS)<sup>[17]</sup>. Participating centers enter data collected on all bariatric surgery patients during preoperative visits, the hospital stay, and all postoperative visits. These data are used to monitor adherence to the requirements of the BSCO program and to support quality assessments for the surgical treatment of obesity and its associated conditions.

All BSCO programs undergo a site inspection before approval and recertification every 3 years. During the site inspection, the accuracy of BOLD data is verified in an impartially selected sample of 10% of medical records. All data on complications and readmissions are also reviewed for accuracy. To ensure accurate data input, any discrepancies result in a mandatory review of all charts at that center.

### Research data

The Copernicus Group Independent Review Board (Durham, NC) approved the use of BOLD data for research. The BOLD study has been registered with the National Institutes of Health (clinical trial #NCT01002352). Informed consent was obtained from all parents and assent was obtained from all patients.

About 65% (169000) of patients treated by surgeons participating in the BSCO program have allowed their data to be analyzed for research purposes. Preliminary analyses show that the demographic characteristics of patients included in the database do not differ substantially from those who are not<sup>[17]</sup>. Data are currently entered into the database by more than 1000 surgeons from more than 600 facilities in the United States (all states are represented with the exception of Vermont and New Mexico), representing approximately 85% of all facilities nationwide performing at least 10 bariatric procedures per year.

### Patient selection

We analyzed data from all patients 11 to 19 years old who had undergone bariatric surgery between April 2004 and October 2010 (the most current data available for analysis) who allowed their data to be used for research purposes. The data analyzed here came from 360 facilities and 423 surgeons participating in the BSCO program.

All patients had met the National Institutes of Health criteria for bariatric surgery<sup>[18]</sup>. Thus, all patients had a BMI > 35 kg/m<sup>2</sup> and ≥ 1 co-morbidity (*e.g.*, elevated blood pressure, hypercholesterolemia) or a BMI > 40 kg/m<sup>2</sup>.

### Data collection

We collected data on age, sex, race/ethnicity, weight (kg), BMI, and weight loss, calculated as the difference between the weight before surgery and the weight at each respective time point.

Intraoperative data used in the present analysis consisted of the procedure (gastric bypass or adjustable gastric band) and the date of surgery. The primary outcomes were weight loss measured by various anthropometric measures and consisted of age- and sex-adjusted BMI percentiles as well as crude BMI and weight (kg)<sup>[19]</sup>. Data were collected before surgery and at 3, 6 and 12 mo after surgery.

Because not all patients had their follow-up appointment exactly at these times, the 3-mo data collection point included data collected from 0 to 3 mo after surgery, the 6-mo time point consisted of data collected from 3 to 9 mo after surgery, and the 12-mo time point consisted of data collected 9 to 15 mo after surgery.

### Statistical analysis

To assess changes in weight and BMI, separate repeated-measures, linear mixed-models were fit using the MIXED procedure in SAS version 9.2 (SAS Institute, Inc., Cary, NC, United States). A compound symmetric variance-covariance matrix was selected for each model to account for the correlation of within-patient repeated observations. Age at surgery, sex, surgery type (gastric bypass or adjustable gastric band), and time period of data collection were the fixed covariates considered for potential inclusion in each model; patients were considered to be a random variable. Because the sample was so skewed in terms of distribution of BMI percentile (the majority of patients had a BMI at or above the 99<sup>th</sup> percentile, the clinical definition of “severe obesity” before surgery) and because the literature consistently reports that comorbidities intensify and multiply with increased weight<sup>[4,5]</sup> we also analyzed the results for three categories defined by baseline BMI values: 35.0-49.0 kg/m<sup>2</sup>; 50.0-59.99 kg/m<sup>2</sup>; and greater than 60.0 kg/m<sup>2</sup>.

Patients may not have been eligible for follow-up analysis if they had surgery shortly before the analysis: 89% of eligible patients were seen at 3 mo ( $n = 739$ ), 62% at 6 mo ( $n = 512$ ), and 30% ( $n = 247$ ) at 12 mo after surgery. Those identified as “other” are not included in the denominator or the follow-up analysis as a result of the small sample size.

Because of potential selection bias caused by losses to follow-up, we tested for any selection bias between the whole sample ( $n = 890$ ) and those patients for which data from all four time-points were available ( $n = 247$ ) using the mixed-model approach described below and by including an indicator variable for complete or incomplete

**Table 1 Characteristics of 890 adolescents who underwent bariatric surgery between 2004 and 2010**

Characteristic	<i>n</i> (%)	Baseline BMI Z score, mean ± SD	<i>P</i>
Sex			< 0.001
Boys	225 (25.3)	3.14 ± 0.24	
Girls	665 (74.7)	2.41 ± 0.19	
Type of surgery			< 0.001
Gastric bypass	454 (51.0)	2.67 ± 0.38	
Adjustable gastric band	436 (49.0)	2.53 ± 0.36	
Race			
Non-hispanic white	606 (68.1)	2.57 ± 0.37	Reference
Hispanic	129 (14.5)	2.68 ± 0.40	0.004 <sup>1</sup>
Non-hispanic black	98 (11.0)	2.67 ± 0.34	0.02 <sup>2</sup>
Other	57 (6.4)	2.60 ± 0.42	

<sup>1</sup>Non-Hispanic white *vs* Hispanic; <sup>2</sup>Non-Hispanic white *vs* non-Hispanic black. BMI: Body mass index.

data<sup>[20]</sup>. Results showed that the two samples did not differ on baseline BMI or weight outcomes or between the proportion of males and females. This finding supports the external validity of our overall conclusions.

In a mixed model, the particular levels of fixed-effects are of interest, and inferences are made for those specific levels; random-effects are considered to be random samples from the population, and inferences are not made to a specific sample but to the entire population. The interaction between time and ethnicity was also assessed. A planned-comparisons approach was used to evaluate changes between ethnic groups because mean baseline BMI values differed significantly. Contrasts were used to test for differences between groups at each time for mean values of weight and BMI. *Alpha* was set at 0.05.

## RESULTS

Of the 890 eligible adolescents, 75% were females (mean age, 18.5 years 68% were white non-Hispanic, 15% were Hispanic, 11% were non-Hispanic black, and 6% were “other” (Table 1). Gastric bypass surgery was performed in 51% of the patients, and gastric band surgery in 49%. At baseline, males were significantly heavier than females. Those undergoing gastric bypass surgery were significantly heavier than those undergoing adjustable gastric band surgery. Non-Hispanic whites were significantly lighter than their ethnic group counterparts (Table 1). Because of these significant baseline differences in sex and surgery type, all subsequent analyses are adjusted for these variables.

All anthropometric measures significantly decreased in non-Hispanic whites and Hispanics throughout the 1 year follow-up period (Table 2). Among both non-Hispanic whites and Hispanics, mean BMI percentile decreased to just below non-obese levels 1 year after surgery (94.67<sup>th</sup> percentiles for both groups, respectively).

When the sample was stratified by sex the variation among ethnic groups was only 3.7 kg for girls and 10 kg for boys (Tables 3 and 4). Further analysis revealed no

**Table 2** Estimated anthropometric values of morbidly obese adolescents at baseline to 1 year after bariatric surgery by ethnicity

Measurement	Non-hispanic whites, estimate (95%CI)	Non-hispanic blacks, estimate (95%CI)	Hispanics, estimate (95%CI)	Overall, estimate <sup>1</sup> (95%CI)
<b>Baseline</b>				
<i>n</i>	601	98	128	827
BMI (kg/m <sup>2</sup> )	47.9 (47.3-48.4)	51.7 (50.1-53.4)	48.9 (47.7-50.2)	48.5 (48.0-49.0)
BMI (%)	99.3 (99.2-99.4)	99.5 (99.4-99.6)	99.4 (99.3-99.5)	99.4 (99.3-99.4)
Weight (kg)	136.8 (135.0-138.6)	147.9 (142.5-153.3)	137.7 (134.1-141.3)	138.4 (136.8-140.0)
<b>3 mo after surgery</b>				
<i>n</i>	536	88	115	739
BMI (kg/m <sup>2</sup> )	43.9 (43.4-44.5)	47.9 (46.4-49.4)	44.5 (43.3-45.6)	44.48 (43.98-44.97)
BMI (%)	99.0 (98.0-99.1)	99.3 (99.2-99.4)	99.1 (99.0-99.2)	99.02 (98.96-99.08)
Weight (kg)	125.6 (123.9-127.4)	137.0 (132.0-141.9)	125.1 (121.7-128.4)	126.84 (125.33-128.35)
EWL (kg)	10.7 (10.1-11.3)	10.57 (9.11-12.04)	12.3 (10.5-14.1)	10.9 (10.4-11.5)
EWL (%)	7.8 (7.3-8.2)	6.9 (6.0-7.8)	8.6 (7.4-9.7)	7.8 (7.4-8.2)
<b>6 mo after surgery</b>				
<i>n</i>	380	54	78	512
BMI (kg/m <sup>2</sup> )	39.0 (38.3-39.7)	44.1 (42.4-45.7)	39.9 (38.5-41.2)	39.70 (39.1-40.3)
BMI (%)	97.0 (96.5-97.5)	98.9 (98.6-99.1)	97.8 (97.3-98.3)	97.3 (96.9-97.7)
Weight (kg)	111.7 (109.7-113.7)	125.7 (120.4-131.0)	111.9 (108.2-115.6)	113.3 (111.6-115.0)
EWL (kg)	25.1 (24.1-26.0)	22.3 (19.7-25.0)	25.4 (22.8-28.0)	24.8 (23.9-25.7)
EWL (%)	18.3 (17.66-19.0)	14.5 (12.9-16.1)	18.1 (16.2-19.9)	17.8 (17.2-18.4)
<b>12 mo after surgery</b>				
<i>n</i>	193	26	28	247
BMI (kg/m <sup>2</sup> )	36.6 (35.7-37.4)	40.3 (37.5-43.0)	36.7 (34.7-38.8)	37.0 (36.3-37.8)
BMI (%)	94.7 (93.6-95.7)	96.8 (94.3-99.2)	94.7 (92.2-97.2)	94.9 (94.0-95.8)
Weight (kg)	104.6 (102.1-107.0)	114.7 (106.4-123.0)	103.1 (97.3-109.0)	105.6 (103.4-107.8)
EWL (kg)	32.8 (30.9-34.7)	33.8 (27.3-40.3)	34.3 (30.0-38.5)	33.1 (31.4-34.82)
EWL (%)	23.6 (22.3-24.9)	21.6 (17.4-25.7)	24.7 (21.8-27.7)	23.5 (22.4-24.7)

Model adjusted by age, sex, and type of surgery; "Other" group not included as result of a small sample size. EWL: Estimated weight loss or the difference between baseline weight (kg) and weight at each respective time point; <sup>1</sup>P < 0.001 for change across all time points. BMI: Body mass index.

**Table 3** Estimated anthropometric values from morbidly obese boys 1 year after bariatric surgery by ethnicity

Measurement	Non-hispanic whites, estimate (95%CI)	Non-hispanic blacks, estimate (95%CI)	Hispanics, estimate (95%CI)	Overall, estimate <sup>1</sup> (95%CI)
<b>Before surgery</b>				
<i>n</i>	142	23	42	207
BMI (kg/m <sup>2</sup> )	51.2 (49.8-52.7)	52.3 (49.0-55.5)	52.8 (50.8-54.9)	51.68 (50.53-52.84)
BMI (%)	99.9 (99.6-100.0)	99.9 (99.8-100.0)	99.9 (99.8-100.0)	99.9 (99.8-100.0)
Weight (kg) <sup>2</sup>	162.4 (158.0-166.7)	166.7 (155.3-178.2)	161.8 (155.8-167.8)	162.8 (159.3-166.3)
<b>3 mo after surgery</b>				
<i>n</i>	127	21	38	186
BMI (kg/m <sup>2</sup> )	46.9 (45.5-48.3)	47.5 (44.8-50.2)	47.1 (45.2-48.9)	46.9 (45.9-48.1)
BMI (%)	99.7 (99.6-99.8)	99.9 (99.7-99.9)	99.8 (99.7-99.9)	99.8 (99.7-99.9)
Weight (kg) <sup>2</sup>	148.5 (144.1-152.8)	151.7 (141.7-161.7)	144.3 (138.6-149.9)	148.0 (144.5-151.4)
EWL (kg) <sup>2</sup>	13.2 (11.7-14.6)	14.4 (10.9-17.9)	17.3 (13.3-21.3)	14.1 (12.8-15.4)
EWL (%)	8.1 (7.3-8.9)	8.5 (6.6-10.3)	10.4 (8.2-12.6)	8.6 (7.9-9.4)
<b>6 mo after surgery</b>				
<i>n</i>	83	17	26	126
BMI (kg/m <sup>2</sup> )	41.9 (40.3-43.9)	43.4 (40.9-45.9)	41.8 (39.9-43.6)	42.0 (40.8-43.2)
BMI (%)	98.7 (98.1-99.3)	99.6 (99.4-99.8)	99.6 (99.4-99.7)	98.9 (98.6-99.4)
Weight (kg) <sup>2</sup>	132.7 (127.8-137.6)	138.5 (129.8-147.1)	128.1 (122.4-133.8)	132.4 (128.7-136.1)
EWL (kg) <sup>2</sup>	29.7 (27.4-31.9)	28.1 (22.8-33.5)	34.0 (29.6-38.4)	30.3 (28.4-32.1)
EWL (%)	18.3 (16.8-19.8)	16.2 (13.9-18.6)	20.5 (18.2-22.8)	18.4 (17.3-19.6)
<b>12 mo after surgery<sup>2,3</sup></b>				
<i>n</i>	49	7	9	65
BMI (kg/m <sup>2</sup> )	39.6 (37.8-41.5)	40.2 (35.5-44.8)	37.0 (34.0-40.0)	39.3 (37.8-40.9)
BMI (%)	97.5 (95.8-99.1)	98.6 (96.9-100.4)	98.2 (96.8-99.7)	97.7 (96.5-98.9)
Weight (kg) <sup>2</sup>	125.3 (119.6-130.9)	127.9 (113.3-142.5)	113.7 (104.6-122.9)	128.7 (118.9-128.5)
EWL (kg) <sup>2</sup>	37.9 (33.7-42.1)	40.4 (26.8-54.0)	47.9 (41.2-54.6)	40.1 (36.5-43.7)
EWL (%)	22.9 (20.4-25.4)	23.0 (16.0-30.0)	29.4 (25.2-33.6)	24.0 (21.9-26.2)

Model adjusted by age and type of surgery; "Other" group not included due to small sample size; <sup>1</sup>P < 0.01 for change across all time points for Hispanics; <sup>2</sup>P < 0.001 for change across all time points; <sup>3</sup>P < 0.001 for change across all time points, except for weight percentile was not significant for non-Hispanic black; EWL: Estimated weight loss or the difference between baseline weight (kg) and weight at each respective time point; BMI: Body mass index.

**Table 4 Mean anthropometric values from morbidly obese girls 1 year after bariatric surgery by ethnicity**

Measurement	Non-hispanic whites, estimate (95%CI)	Non-hispanic blacks, estimate (95%CI)	Hispanics, estimate (95%CI)	Overalls, Estimate <sup>1</sup> (95%CI)
Before Surgery				
<i>n</i>	459	75	86	620
BMI (kg/m <sup>2</sup> )	46.8 (46.2-47.5)	51.6 (49.6-53.6)	47.0 (45.5-48.6)	47.5 (46.9-48.1)
BMI (%)	99.1 (99.0-99.2)	99.4 (99.3-99.5)	99.2 (99.0-99.3)	99.2 (99.1-99.3)
Weight (kg)	129.1 (127.18-131.1)	141.6 (135.4-147.7)	125.9 (121.5-130.4)	130.3 (128.6-132.1)
3 mo after surgery				
<i>n</i>	409	67	77	553
BMI (kg/m <sup>2</sup> )	43.0 (42.4-43.6)	48.0 (46.3-49.8)	43.3 (41.8-44.7)	43.7 (43.1-44.2)
BMI (%)	98.7 (98.6-98.8)	99.1 (98.9-99.2)	98.7 (98.5-98.9)	98.8 (98.7-98.9)
Weight (kg)	118.7 (116.9-120.5)	131.9 (126.3-137.6)	116.0 (111.8-120.2)	119.9 (118.2-121.6)
EWL (kg)	9.9 (9.3-10.7)	9.2 (7.6-10.7)	9.8 (8.0-11.6)	9.8 (9.2-10.4)
EWL (%)	7.7 (7.1-8.2)	6.3 (5.3-7.4)	7.7 (6.3-9.1)	7.5 (7.0-7.9)
6 mo after surgery				
<i>n</i>	297	37	52	386
BMI (kg/m <sup>2</sup> )	38.1 (37.4-38.8)	44.4 (42.3-46.4)	39.0 (37.1-40.8)	38.9 (38.3-39.6)
BMI (%)	96.5 (95.9-97.1)	98.6 (98.3-98.9)	96.9 (96.2-97.7)	96.8 (96.3-97.2)
Weight (kg)	105.3 (103.2-107.4)	121.8 (115.4-128.3)	104.2 (99.3-109.1)	107.0 (105.1-108.8)
EWL (kg)	23.7 (22.6-24.8)	20.0 (16.9-23.2)	21.2 (17.9-24.5)	22.9 (21.9-24.0)
EWL (%)	18.3 (17.5-19.1)	13.8 (11.7-15.8)	16.9 (14.4-19.5)	17.6 (16.9-18.4)
12 mo after surgery				
<i>n</i>	149	19	19	182
BMI (kg/m <sup>2</sup> )	35.6 (34.7-36.6)	40.32 (36.96-43.67)	36.65 (34.15-39.16)	36.28 (35.40-37.17)
BMI (%)	93.9 (92.6-95.1)	96.10 (92.81-99.39)	92.99 (89.48-96.51)	93.99 (92.87-95.12)
Weight (kg)	98.3 (95.5-101.0)	110.4 (100.5-120.2)	98.0 (91.3-104.6)	99.6 (97.1-102.1)
EWL (kg)	31.2 (29.1-33.4)	31.4 (24.0-38.8)	27.8 (22.9-32.6)	30.9 (28.9-32.8)
EWL (%)	23.8 (22.3-25.2)	21.1 (16.1-26.2)	22.5 (18.7-26.3)	23.4 (22.0-24.7)

Model adjusted for age and type of surgery; "Other" group not included due to small sample size; <sup>1</sup>*P* < 0.001 for change across all time points. EWL: Estimated weight loss or the difference between baseline weight (kg) and weight at each respective time point; BMI: Body mass index.

**Table 5 Mean change in anthropometric values from before to 1 year after bariatric surgery by ethnicity**

Measure	Estimated change 12 mo after surgery (mean ± SE)				<i>P</i>		
	NHW	NHB	Hispanic	Overall	NHW vs NHB	NHW vs Hispanic	NHB vs Hispanic
BMI (kg/m <sup>2</sup> )	-11.4 ± 0.5	-11.2 ± 1.6	-12.7 ± 1.2	-11.5 ± 0.4	0.87	0.29	0.41
BMI (%)	-4.7 ± 0.6	-2.7 ± 1.3	-4.9 ± 1.3	-4.6 ± 0.5	0.2	0.89	0.29
Weight (kg)	-32.2 ± 1.3	-32.3 ± 4.6	-36 ± 3.5	-32.6 ± 1.2	0.89	0.31	0.44

Model adjusted for age, sex, and type of surgery. BMI: Body mass index; NHW: Non-hispanic white; NHB: Non-hispanic black.

**Table 6 Effect of baseline body mass index on weight loss 1 year after bariatric surgery**

Variable	Baseline BMI category		
	Lowest (35-49 kg/m <sup>2</sup> )	Middle (50-59 kg/m <sup>2</sup> )	Highest (> 60 kg/m <sup>2</sup> )
Change in BMI, kg/m <sup>2</sup> (%) <sup>1</sup>	-9.88 (-22.6)	-14.34 (-26.5)	-17.8 (-26.7)
Rate of change over the year (kg/y)			
Non Hispanic whites <sup>2</sup>	-9.6	-14.8	-19.7
Hispanics	-11.7	-11.7	-11.6
Non Hispanic blacks	-9.3	-11.1	-15.5

<sup>1</sup>*P* < 0.0001 for lowest versus middle, lowest versus highest, and middle versus highest BMI category; <sup>2</sup>*P* < 0.0001 for lowest versus middle, lowest versus highest, and *P* = 0.002 for middle versus highest BMI category. BMI: Body mass index.

significant differences between pairwise ethnic group comparisons for all anthropometric measures (Table 5).

Changes in BMI varied somewhat by preoperative BMI category. For the entire sample, the highest BMI category lost the most BMI units when compared to lowest BMI category (17.8 kg/m<sup>2</sup> vs 9.9 kg/m<sup>2</sup>, respectively, *P* < 0.001). Only Hispanics showed no difference in weight loss between the three groups (11.7 kg/m<sup>2</sup>) (Table 6). Rate of change over the year for the three BMI categories did not vary much by ethnicity or sex, but they did by surgery type: in all three categories, BMI was reduced more among those who underwent gastric bypass surgeries than among those who had adjustable band surgeries (*P* < 0.001) (data not shown on tables).

Details about aggregate post-operative complications are published elsewhere<sup>[9]</sup>. Briefly, of the 120 complications reported (13.5%), 38 were with the gastrointestinal system and 26 were with nutritional deficiencies. Complication rates did not differ by ethnicity. Similarly, when readmissions (*n* = 55) were analyzed by ethnic group, no significant differences were found. (Data not reported).

One death was reported from cardiac arrest 5 mo after (gastric bypass) surgery.

## DISCUSSION

In a national sample of ethnically diverse, severely obese adolescents, 1 year after bariatric surgery, weight and other anthropometric measures had decreased by the same amount in all racial/ethnic groups and in both sexes. Mean estimated weight loss for all three groups at 1 year differed by a maximum of only 1.5 kg. Surgery resulted in an average weight loss of more than 30 kg per person, a loss that far exceeds those reported in non-surgical weight-management programs<sup>[21]</sup>.

Among adults, ethnicity predicts better weight loss outcomes after bariatric surgery, with some studies reporting better outcomes among NHWs than among NHBs. There is little information about outcomes in Hispanics. Harvin *et al*<sup>[13]</sup> found that 2 years after undergoing bariatric surgery, NHW adults had lost significantly more weight than did their ethnic group counterparts. Similarly, Buffington *et al*<sup>[15]</sup> found that NHB adults had less-successful weight outcomes after gastric bypass than did NHW adults. Among adults, NHB women lost considerably less weight after surgery than did NHW women<sup>[13-16]</sup>. Additionally, in the Netherlands, Admiraal *et al*<sup>[22]</sup> found that African, South Asian, Turkish and Moroccan patients lost less weight at 1-year post-gastric bypass surgery versus their ethnic Dutch counterparts.

Conversely, most studies of adolescent bariatric surgery are case series from a single-institution, resulting in samples that are generally small and relatively homogeneous, which does not permit robust comparisons among ethnic groups<sup>[8]</sup>. Although race and ethnicity are independently associated with cardiometabolic disease risk<sup>[23,24]</sup>, we found that weight loss was similar among all three ethnic groups and varied more among boys than among girls. However, no overall pairwise group comparisons were significant, indicating that no ethnic group had better weight loss outcomes than did another, unlike comparisons of adults, as described above. Parental influence over post-operative adherence to quality nutrition and physical activity recommendations may partially explain the lack of ethnic group differences.

Although the childhood obesity epidemic continues unabated in most developed countries, non-surgical approaches to the long-term (1 year or more) management and decrease of overweight in childhood have had limited success<sup>[21]</sup>. Despite standardized indications for bariatric surgery in adolescents<sup>[25]</sup>, obese children are not simply younger versions of obese adults; they are still developing and growing, both physically and psychologically. Extreme obesity should be treated sooner rather than later<sup>[8]</sup>, particularly in adolescents, who may have not yet developed full-blown, related comorbidities, such as diabetes or heart disease. Our analysis found that type of surgery significantly influences weight loss, however and thus must be a consideration for adolescents considering this

alternative; gastric bypass surgery resulted in significantly more weight loss at 1-year versus adjustable gastric band surgery. Therefore, gastric bypass may be a viable option for those who are in need of more weight loss to resolve co-morbidities that have already developed. However, the optimal age in adolescence for bariatric surgery is as yet undetermined<sup>[24]</sup>. Our findings, and those of others<sup>[26,27]</sup> indicate that bariatric surgery before adulthood can substantially reduce weight<sup>[10,25]</sup> and resolve comorbidities<sup>[25]</sup>. Moreover, earlier treatment of obesity may prevent later costs. For example, children and adolescents with a primary or secondary diagnosis of overweight, obesity, or severe obesity require longer hospital stays than do children without these diagnoses<sup>[28]</sup>.

Even as the emerging data on bariatric surgery—including those from randomized-controlled trials<sup>[26,29]</sup>—continue to show important long-term weight loss and improvement in most obesity-related comorbidities, many pediatric specialists still hesitate to refer patients for surgery<sup>[30]</sup>. A survey of several hundred pediatricians in the United States showed that, although they believed pediatric obesity to be a major problem, less than half would be somewhat or very likely to refer a severely obese adolescent for surgery<sup>[30]</sup>. Yet, the medical consequences of childhood obesity continue and suggest that overt disease beginning in early adulthood may become chronic<sup>[31]</sup>.

## Limitations of the study

Our findings and conclusions are limited by a substantial amount of missing follow-up data, a common problem in the bariatric literature among both adults and adolescents. Older adolescents in particular are difficult to follow because they may leave the geographic area for education or employment. More age-relevant tracking procedures, such as those based on social media or handheld or cellular telephone devices, may be able to decrease losses to follow-up.

Variations in practice management among BSCOPE participants may delay data entry, potentially resulting in incomplete follow-up data. Additionally, pre-surgical information on nutritional deficiencies is not available to determine whether surgery is the cause of the few deficiencies we found or whether these deficiencies existed before surgery.

Our results support the conclusion that bariatric surgery can substantially reduce weight in severely obese adolescents for at least 1 year, irrespective of their race or ethnicity. Ethnicity should not be a contraindication for bariatric surgery in adolescents, which is a reasonable and safe treatment for all severely obese adolescents with the appropriate indications.

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their data for the current analyses.

## COMMENTS

### Background

Several studies have shown that among adults, weight loss outcomes after bariatric surgery differ by ethnicity. Although the literature on outcomes of bariatric surgery in adolescents has increased exponentially, many studies are small and usually from single institutional series. As a result, patients are not geographically or ethnically diverse, despite the fact that obesity disproportionately affects ethnic minorities.

### Research frontiers

Bariatric surgery is one of the few effective treatments for morbid obesity. Among adults, outcomes of bariatric surgery differ by ethnicity.

### Innovations and breakthroughs

In a national sample of ethnically diverse, severely obese adolescents, 1 year after bariatric surgery, weight and other anthropometric measures had decreased by the same amount in all racial/ethnic groups and in both sexes. Mean estimated weight loss for all three groups at 1 year differed by a maximum of only 1.5 kg. Surgery resulted in an average weight loss of more than 30 kg per person, a loss that far exceeds those reported in non-surgical weight-management programs.

### Applications

Bariatric surgery substantially reduces the weight of severely obese adolescents at 1 year post-procedure with little variation by ethnicity and/or gender. These results suggest that bariatric surgery is a safe and reasonable treatment for all severely obese adolescents with the appropriate indications.

### Terminology

Bariatric surgery is synonymous with weight loss surgery and in this analysis consisted of two specific types: gastric bypass surgery and adjustable gastric band surgery.

### Peer review

Attrition is one of the major challenges with this patient population and that is indeed apparent in the analysis here. As a result, the authors could not simultaneously dichotomize surgery type and ethnicity thus why surgeries are reported in aggregate. Instead, the purpose of this article was to focus on exploring ethnic group differences in weight loss 1 year after surgery.

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## Impaired fasting glucose: Pro-diabetic, "atheroprotective" and modified by metabolic syndrome

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### Abstract

**AIM:** To investigate whether impaired fasting glucose (IFG) confers cardiovascular risk.

**METHODS:** A non-diabetic population-based sample representative of middle-aged and elderly Turks was studied at 8.5 years' follow-up for incident diabetes and coronary heart disease (CHD). Metabolic syndrome (MetS) was defined by ATP-III criteria modified

for male abdominal obesity, and IFG and type 2 diabetes were identified by criteria of the American Diabetes Association. Stratification by presence of MetS was used. Outcomes were predicted providing estimates for hazard ratio (HR) obtained by use of Cox proportional hazards regression analysis in models that controlled for potential confounders.

**RESULTS:** In 3181 adults (aged  $52 \pm 11.5$  years at baseline), analysis stratified by MetS, gender and IFG status distinguished normoglycemic subjects by a "hypertriglyceridemic waist" phenotype consisting of significantly higher waist circumference, fasting triglyceride and lower high-density lipoprotein-cholesterol, regardless of gender and MetS. Additionally, lipoprotein (Lp) (a) tended to be lower in (especially female) participants with MetS. Multivariable linear regression in a subset of the sample demonstrated decreased Lp (a) levels to be associated with increased fasting glucose and insulin concentrations, again particularly in women. In Cox regression analysis, compared with normoglycemia, baseline IFG adjusted for major confounders significantly predicted incident diabetes at a 3-fold HR in men and only women with MetS. Cox models for developing CHD in 339 individuals, adjusted for conventional risk factors, revealed that IFG status protected against CHD risk [HR = 0.37 (95%CI: 0.14-0.998)] in subjects free of MetS, a protection that attenuated partly in male and fully in female participants with MetS.

**CONCLUSION:** IFG status in non-diabetic people without MetS displays reduced future CHD risk, yet is modulated by MetS, likely due to autoimmune activation linked to serum Lp (a).

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**Key words:** Autoimmune activation; Coronary disease risk; Diabetes, type 2; Impaired fasting glucose; Lipoprotein (a); Metabolic syndrome

**Core tip:** The study investigated whether and to what extent impaired fasting glucose (IFG) conferred risk of type 2 diabetes or coronary heart disease in 3181 middle-aged adults, by separately stratifying to gender and metabolic syndrome. Follow-up over 8.5 years revealed both factors to modulate future risk. In women without metabolic syndrome, IFG was not associated with either cardiometabolic disorder, while in men, IFG imparted risk of diabetes alone. Coronary heart disease risk appeared to depend on the core components of the metabolic syndrome, especially in the female. The results implicate autoimmune activation involving lipoprotein(a) to be of high relevance in the pathogenesis of new-onset diabetes.

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## INTRODUCTION

Fasting blood glucose is one of the five components of metabolic syndrome (MetS), the prevalence and the impact on cardiometabolic risk of which is on the rise worldwide. Fasting glucose has been reported to be logarithmically associated with risk of vascular disease in non-diabetic people although related data are inconclusive<sup>[1,2]</sup>. Impaired fasting glucose (IFG), one of the two pre-diabetic states based on an underlying insulin resistance and impaired  $\beta$ -cell function, is recognized to carry excess risk for diabetes but clearly less risk than impaired glucose tolerance (IGT) for cardiovascular risk that is no more than modest<sup>[3,4]</sup>. The meta-analysis by the Emerging Risk Factors Collaboration on nearly 280000 non-diabetic individuals showed that fasting glucose concentration was modestly and non-linearly associated with risk of vascular disease<sup>[1]</sup>. Compared with fasting glucose concentrations of 70-100 mg/dL, HR for coronary heart disease (CHD) were 1.07 for lower than 70 mg/dL; 1.11 for 100-110 mg/dL; and 1.17 for 110-126 mg/dL. IFG status did not significantly improve metrics of vascular disease prediction when added to information about several conventional risk factors<sup>[1]</sup>. Heterogeneity in screened populations is suggested by individual studies that have reported no excess cardiovascular risk<sup>[5-8]</sup>. A meta-analysis of clinical trials on interventions in subjects with pre-diabetes did not show reductions in all causes or cardiovascular mortality, contrasted to preventing progression to overt diabetes<sup>[9]</sup>.

We have confirmed, among nearly 2900 Turkish adults, increased multi-adjusted (J-shaped) risk of incident CHD for fasting glucose category < 90 mg/dL, compared with the category 90-110 mg/dL<sup>[10]</sup>, an obser-

vation that was at variance with one meta-analysis<sup>[11]</sup> but in agreement with another<sup>[1]</sup>. There was evidence that higher CHD risk observed in low compared to higher normal glucose concentrations was likely related to an associated pro-inflammatory state that required further investigation.

However, these analyses did not discriminate the role of the presence of MetS regarding the impact of IFG on CHD risk. Our previous observations indicated that age-adjusted MetS prevailed more in people with the lowest than the intermediate lipoprotein (Lp) (a) tertile<sup>[12]</sup>, and an 11% mean decline in sex- and age-adjusted Lp (a) levels was reported in individuals with diabetes<sup>[13]</sup>, which is usually associated with activation of complement pathways.

We, therefore, aimed to examine the impact of IFG simultaneously on incident type 2 diabetes and CHD in non-diabetic middle-aged Turkish adults of the general population by stratifying to the presence of MetS lending care to potential sex differences that had been shown in Turkish adults with respect to cardiometabolic risk<sup>[14,15]</sup>. The association of serum Lp (a) and apolipoprotein apoA-I with fasting glucose was also investigated in a subset of the study sample. Such an approach yielded the novel information that IFG, although conferring excess diabetes risk, represented an atheroprotective status compared with normoglycemia in non-diabetic people without MetS, and involvement of Lp (a) in autoimmune activation might be an underlying factor.

## MATERIALS AND METHODS

### Population sample

The Turkish Adult Risk Factor study is a longitudinal population-based cohort study on cardiac disease and its risk factors in adults in Turkey, carried out biennially in 59 communities in all geographical regions<sup>[16]</sup>. It involves a random sample of the Turkish adult population, representatively stratified for sex, age, geographical regions and for rural-urban distribution<sup>[16]</sup>. Combined measurements of waist circumference and high-density lipoprotein (HDL) cholesterol being first available at the follow-up visit in 1997-1998, the latter examination formed the baseline. Participants, 28 years of age or older at baseline, were examined periodically up to the survey 2010-2011. When individuals with prevalent diabetes and missing values for fasting (or 2-h postprandial) glucose at baseline were excluded, the remaining 3181 non-diabetic participants formed the cohort of the current study. The survey conformed to the principles embodied in the Declaration of Helsinki and was approved by the Istanbul University Ethics Committee. Individuals of the cohort gave written consent for participation. Data were obtained by history of the past years *via* a questionnaire, physical examination of the cardiovascular system, sampling of blood and recording of a resting 12-lead electrocardiogram.

### Measurements of risk variables

Blood pressure (BP) was measured using a sphygmo-

manometer (Erka, Bad Tölz, Germany) after at least 5 min of rest while seated, and the mean of two recordings was recorded. Waist circumference was measured with a tape (Roche LI95 63B 00), the subject standing, at the level midway between the lower rib margin and the iliac crest. Body mass index (BMI) was computed as weight divided by height squared ( $\text{kg}/\text{m}^2$ ). Self-reported cigarette smoking was categorized into never smokers, former smokers (discontinuance of 3 mo or more) and current smokers (regularly 1 or more cigarettes daily).

Plasma concentrations of total and high-density lipoprotein (HDL) cholesterol, ( $> 11$  h) fasting triglycerides and glucose were determined at baseline examination by the enzymatic dry chemistry method using a Reflotron apparatus. Low-density lipoprotein (LDL) cholesterol values were computed according to the Friedewald formula. In the final five surveys, the stated parameters, as well as insulin and C-reactive protein (CRP) values, were assayed in a single central laboratory. Blood samples were shipped to Istanbul and stored in deep-freeze at  $-75^\circ\text{C}$  until analyzed. Concentrations of insulin were determined by the electrochemiluminescence immunoassay ECLIA on Roche Elecsys 2010 (Roche Diagnostics, Mannheim, Germany). Serum concentrations of apoA-I, apoB, apoE, and Lp (a) were measured by the Behring nephelometry (Behring Diagnostics, Marburg, Germany). Serum  $\gamma$ -glutamyltransferase activity was assayed by the kinetic method using Glucana as substrate (Thermo Trace, Noble Park, Victoria, Australia). External quality control was performed with a reference laboratory in a random selection of 5%-6% of participants. Data on baseline fasting insulin, Lp (a) and apoA-I were available in 71%, 50% and 34% of participants, respectively.

### Definitions and outcomes

Subjects in whom plasma glucose was measured after breakfast outside a 1.5-2.5 h period were not included. As recommended by the American Diabetes Association, individuals with IFG were identified with fasting glucose concentrations of 5.56-6.98 mmol/L, or (in one-fifth of the sample) with levels of 5.60-7.78 mmol/L 1.5-2.5 h after breakfast; type 2 diabetes with  $\geq 7.0$  mmol/L (or 2 h postprandial glucose  $\geq 11.1$  mmol/L) and/or the current use of diabetes medication<sup>[17]</sup>. MetS was identified when 3 out of the 5 criteria of the National Cholesterol Education Program ATP-III were met, modified for pre-diabetes (fasting glucose 5.56-6.95 mmol/L)<sup>[18]</sup> and further for male abdominal obesity using as a cut point  $\geq 95$  cm<sup>[19]</sup>, as assessed in the Turkish Adult Risk Factor study. Biological evidence of functional defectiveness of HDL and apoA-I particles as discerned by outcomes of diabetes and/or CHD has been designated as HDL dysfunction<sup>[15,20,21]</sup>.

Diagnosis of nonfatal CHD was based on the presence of angina pectoris, of a history of myocardial infarction with or without accompanying Minnesota codes of the electrocardiogram (ECG)<sup>[22]</sup>, or on a history of myocardial revascularization. Typical angina and age

$> 45$  years in women were prerequisite for a diagnosis when angina was isolated. ECG changes of "ischemic type" of greater than minor degree (codes 1.1-2, 4.1-2, 5.1-2, 7.1) were considered as myocardial infarct sequelae or myocardial ischemia, respectively. Cause of death was assigned in accordance with the information on the mode of death obtained from first degree relatives and/or local health personnel, also considering pre-existing clinical and laboratory findings elicited during biennial surveys.

### Statistical analysis

Descriptive parameters were shown as mean  $\pm$  SD or in percentages. Due to skewed distribution, values derived from log-transformed (geometric) means were used for serum triglycerides, insulin, apoE and Lp (a). Multiple linear regression analysis was performed with continuous parameters using Lp (a) as a dependent variable and apoA-I/HDL-C ratio as an independent variable. This ratio was considered to potentially reflect greater proportional increase in serum apoA-I than HDL-C, in line with the notion of  $\text{pre}\beta$ -1 HDL<sup>[23]</sup>. In predicting outcomes at baseline examination in multivariate analyses, estimates (and 95%CI) for hazard ratio (HR) were obtained by use of Cox proportional hazards regression analysis in models that controlled for potential confounders. HRs were expressed in terms of 1 SD increment. A value of  $P < 0.05$  on the two-sided test was considered statistically significant. Statistical analyses were performed using SPSS-10 for Windows (SPSS Inc., Chicago, IL, United States, Nr. 9026510).

## RESULTS

Mean age of the study sample at baseline (1564 men and 1617 women) was  $52.0 \pm 11.5$  years. Mean follow-up with respect to diabetes amounted to  $8.5 \pm 3.5$  years (range 2-13 years) and to CHD  $8.4 \pm 3.7$  years (range 1-13 years). Diabetes developed at a mean rate of 11.3 per 1000 person-years, CHD at 15.0 per 1000 person-years.

Normoglycemic men without MetS were younger, had a wider waist, higher triglycerides, LDL and total cholesterol, and lower HDL-C than men with IFG but without MetS (Table 1). They tended also to have higher apoB levels. Normoglycemic women without MetS had a wider waist, higher triglycerides and lower HDL-C, and tended to lower Lp (a) than women with IFG yet free of MetS. This suggests that aggregation between Lp (a) and a protective protein was in operation, enhancing the pro-inflammatory state.

Normoglycemic men with MetS were younger, had a wider waist, higher triglycerides and creatinine, and lower HDL-C than men with IFG and MetS. Normoglycemic women with MetS had a wider waist, higher triglycerides, LDL and total cholesterol, diastolic BP, lower HDL-C levels and tended to higher apoE, apoB and lower apoA-I than women with IFG and MetS.

Table 1 Baseline characteristics of the study sample stratified by gender, presence of MetS and fasting blood glucose category

	Without metabolic syndrome															
	Men				Women				Men				Women			
	<i>n</i>	N'glyc. 91.4 IFG, 110.5 mg/dL	N'glyc. 92	ANOVA	<i>n</i>	IFG, 109 mg/dL	N'glyc. 93.3	ANOVA	<i>n</i>	IFG, 113.5 mg/dL	N'glyc. 93.3	ANOVA	<i>n</i>	IFG, 111 mg/dL	N'glyc. 93.3	ANOVA
Age (yr)	1826	50.8 ± 12.7	54.3 ± 14.2	0.03	813	49.7 ± 12.2	49.7 ± 11.9	0.99	1355	54.1 ± 11.4	57 ± 12	0.008	471	55.3 ± 11.3	56.2 ± 12.2	0.31
Waist circumference (cm)	1826	89.3 ± 9.6	85.4 ± 8.5	0.001	813	84.7 ± 11	81.7 ± 9.8	0.023	1289	101.1 ± 8.4	98.9 ± 9.3	0.004	471	98.3 ± 10.3	95 ± 12	< 0.001
F: triglycerides (mg/dL)	1336	111.6 ± 16.0	93 ± 1.33	< 0.001	813	95.7 ± 1.48	83.9 ± 1.36	0.001	1043	195 ± 1.62	155.2 ± 1.67	< 0.001	471	164.7 ± 1.61	143 ± 1.65	0.001
HDL cholesterol (mg/dL)	1826	40.5 ± 12.5	43.9 ± 12.3	0.021	813	48.5 ± 13	52.3 ± 13.8	0.017	1291	32.3 ± 7.7	35 ± 10.1	0.002	471	39.4 ± 9	42.1 ± 11.7	< 0.001
LDL cholesterol (mg/dL)	1826	113 ± 30.9	102.8 ± 25.9	0.003	813	116 ± 33.8	109.1 ± 35.1	0.11	1355	116 ± 32.5	115.5 ± 33	0.90	471	127.4 ± 34.3	120.2 ± 34	0.015
Total cholest. (mg/dL)	1826	180 ± 36.5	167.4 ± 30.2	0.003	813	185 ± 38	179 ± 36.5	0.18	1355	189.2 ± 37.7	186.1 ± 35.8	0.35	471	199 ± 38.2	195 ± 39.7	0.18
Apo B (mg/dL)	618	111.8 ± 35	100.7 ± 22.7	0.07	813	108.9 ± 32.7	115 ± 41.6	0.34	505	128.6 ± 38.4	120.8 ± 43.5	0.18	471	131.6 ± 50.2	122.5 ± 41.7	0.13
Apo A-I (mg/dL)	583	127.7 ± 29.7	127.7 ± 35.8	1.00	813	145.9 ± 32	150.7 ± 35.2	0.44	486	123 ± 28.3	124.8 ± 30.8	0.67	471	137 ± 30	145 ± 36.4	0.067
Apo E (mg/dL)	574	4.26 ± 3.73	3.36 ± 0.98	0.24	813	4.03 ± 1.25	4.01 ± 1.11	0.94	455	4.50 ± 2.11	4.67 ± 2.99	0.64	471	4.67 ± 1.97	4.21 ± 1.39	0.063
Fast insulin (mIU/L)	1310	7.14 ± 2.06	5.97 ± 2.23	0.063	813	7.52 ± 2.01	7.41 ± 1.87	0.88	952	10.4 ± 1.80	9.7 ± 1.92	0.28	471	10.3 ± 1.75	9.5 ± 1.93	0.13
Lipoprotein(a) (mg/dL)	884	9.14 ± 2.92	8.48 ± 2.67	0.70	813	10.98 ± 2.93	15.96 ± 3.25	0.073	687	7.75 ± 3.01	8.75 ± 2.67	0.39	471	10.23 ± 2.96	11.32 ± 3.1	0.39
Diastolic BP (mmHg)	1826	77.1 ± 11	76.1 ± 7.8	0.43	813	78.6 ± 12.6	77 ± 12.3	0.18	1355	88 ± 13	87 ± 14.6	0.44	471	90.3 ± 13.5	87.7 ± 14.3	0.013
γ-glutamyltransf (U/L)	984	24.7 ± 1.78	22.5 ± 1.94	0.32	813	16.5 ± 1.80	19.2 ± 1.80	0.13	750	29.7 ± 1.85	27.6 ± 1.84	0.33	471	19.9 ± 1.88	21.1 ± 1.93	0.40
Creatinine (mg/dL)	1060	0.977 ± 0.18	0.963 ± 0.17	0.63	813	0.783 ± 0.31	0.75 ± 0.12	0.51	745	1.04 ± 0.25	0.96 ± 0.18	0.64	471	0.808 ± 0.19	0.77 ± 0.14	0.023
Smoking: curr., fast (%)	1800	58.9 ± 18.9	55.3 ± 21.1	0.82	813	21.9 ± 4	26.3 ± 6.6	0.33	1331	46 ± 25.6	37.7 ± 27.5	0.82	471	11.5 ± 3.8	15.7 ± 4.3	0.24

IFG: Impaired fasting glucose; BP: Blood pressure; Apo: Apolipoprotein; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; ANOVA: Analysis of variance.

**Differences in Lp (a) in subjects with MetS**

In men, while no material difference in circulating Lp (a) existed in individuals with MetS and IFG compared to men free of MetS, concentrations were lower in normoglycemic MetS by 15% ( $P = 0.069$ ) (Table 1). In women, whereas serum Lp (a) was lower in normoglycemia by 7% ( $P = 0.41$ ), concentrations were lower by 29% ( $P = 0.14$ ) in IFG. The data tend to indicate a reduction of Lp (a) concentrations in established MetS.

Multiple linear regression analysis in separate sexes with Lp (a) as a dependent and age, glucose, insulin and apoA-I/HDL cholesterol ratio as independent variables showed inverse associations with age and fasting glucose ( $P < 0.02$ ) in women and a tendency to inverse associations in both sexes with insulinemia (Table 2).

**Cox regression analyses for future outcomes**

Compared with normoglycemia, IFG predicted incident type 2 diabetes with an HR of 2.83 (95%CI: 2.17-3.71), after adjustment for sex, age, BMI, smoking status, non-HDL and HDL cholesterol, and usage of antihypertensive and statin medication (Table 3). BMI alone among the latter confounders contributed significantly, albeit at a lesser magnitude than IFG, towards predicting diabetes. In individuals free of MetS (57% of the sample), women differed from the whole group in as much as IFG did not confer diabetes risk against which current smoking tended to protect, whereas in men the difference concerned statin usage which was associated with future diabetes risk (corresponding to an absolute risk of 1 excess diabetes case for every 145 male users free of MetS).

Table 4 displays Cox proportional hazard models for incident CHD risk stratified to gender and presence of MetS, and comprised of sex, age, systolic BP, smoking status, non-HDL, HDL cholesterol and lipid-lowering drug usage as well. Adults free of MetS but with IFG displayed a clearly reduced HR 0.37 (95%CI: 0.14-0.998) compared with normoglycemic subjects. In participants with MetS, this reduced risk was attenuated in men to an HR of 0.75 and was abolished in women. Aside from age, non-HDL cholesterol (especially in people free of MetS) and systolic BP (especially in men) were major determinants of incident CHD.

**Table 2** Linear regression analysis for serum lipoprotein(a), by gender

	Men (n = 165)			Women (n = 173)		
	$\beta$	SE	P value	$\beta$	SE	P value
Age (11 yr)	1.05	1.11	0.59	0.78	1.01	0.007
Fasting glucose (24 mg/dL)	0.82	1.12	0.14	0.66	1.18	0.018
Fasting insulin (mIU/L)	0.82	1.28	0.43	0.75	1.32	0.31
Apo A-I/HDL-cholesterol ratio (1 U)	1.06	1.09	0.48	1.08	1.09	0.40
	$R^2 = 0.01$ ; $P = 0.45$			$R^2 = 0.06$ ; $P = 0.014$		

Log-transformed values. In the model with sexes combined, female sex ( $\beta = 1.38$ -fold,  $P = 0.009$ ) and glucose ( $\beta = 0.79$ -fold,  $P = 0.022$ ) were significant. Apo: Apolipoprotein; HDL: High-density lipoprotein.

## DISCUSSION

In this sample representative of middle-aged non-diabetic Turkish adults, yet prone to MetS, salient findings were normoglycemic participants (without or with) MetS exhibited a more adverse risk profile than those with ADA-defined IFG (specifically regarding waist circumference, triglyceride and HDL cholesterol); fasting glucose levels (especially in women) were associated with serum Lp (a) inversely and independently of fasting insulin concentrations; IFG status conferred a risk of diabetes, as anticipated, 3-fold as high compared with normoglycemic participants, but did not impart future risk for CHD in subjects with MetS and even protected significantly in those free of MetS. Novel findings elicited *via* such stratified analyses may be attributed in normoglycemic individuals without MetS to the intriguing operation of a tendency to a decline in serum Lp (a), presumably associated with elevation of fasting glucose, while lower levels (particularly in women with IFG) reflect autoimmune activation and, in turn, induce increased risk for MetS and CHD.

### Hypertriglyceridemic waist phenotype, an early feature in people free of MetS

Stratification by gender and presence of MetS clearly and consistently showed significant differences in each of the four groups across normoglycemic and pre-diabetic people, specifically in regard to waist circumference, fasting triglyceride and HDL cholesterol levels. This indicates that the hypertriglyceridemic waist (HtgW) phenotype, together with low HDL-C is the main feature distinguishing normoglycemic from pre-diabetic adults, a remarkable and *a priori* unexpected finding. This finding may, however, be explained by presuming changes in circulating Lp (a) to be the primary determinant in individuals without MetS, which may have mediated changes in fasting plasma glucose. Indeed, Lp (a) was independently linearly associated inversely with fasting insulin concentrations<sup>[12]</sup> (approximately 2% lower Lp (a) per 2-fold increment of insulin). Irrespective of MetS in men, higher insulin levels (by 15%) existed in normoglycemia than in IFG, a constellation noted in women to a lesser magnitude (by 5%).

### Lower Lp (a) concentrations reflect increased insulin resistance

Our multiple regression analysis model for serum Lp (a), albeit in a relatively small subgroup of 338 subjects due to missing values of apo A-I, Lp (a) and fasting insulin, yielded valuable information with respect to independent inverse associations in women of lower Lp (a) with increasing age and higher glycemia. This finding not only supports a notion of autoimmune activation whereby part of Lp (a) protein becomes non-assayable, but also may account for the findings of diminished levels of Lp (a) in type 2 diabetes<sup>[13]</sup> and MetS<sup>[12]</sup>. Lower Lp (a) in non-diabetic females with increasing age further suggests consumption of Lp (a) protein during the slow process of autoimmune complex formation.

### IFG confers no CHD risk although it predicts diabetes in women with MetS and in men

The present study demonstrated nearly a 3-fold diabetes risk for IFG in male participants and females with MetS, after adjustment for variables often used in major diabetes algorithms. This is essentially in line with reports on other populations<sup>[24-26]</sup>, although several validated risk scores do not include IFG as a parameter<sup>[26-28]</sup>.

Yet, IFG was associated with substantially lower future CHD risk than normoglycemia in all men, and women free of MetS. IFG tended to confer slight CHD risk only in women with MetS (*i.e.*, abdominal obesity, atherogenic dyslipidemia and associated pro-inflammatory state). An analogous situation is related to risk of chronic kidney disease (CKD): IFG was the only component of MetS not significantly predicting it in a meta-analysis of 11 studies<sup>[29]</sup>.

### Sex difference

At variance from women, normoglycemic men persist to carry a more adverse risk profile than male IFG, presumably in the process of the development of MetS, along with a slow decline in serum Lp (a), a process congruent with normoglycemia still with a non-significantly higher CHD risk than IFG. A further difference is the lack of change in Lp (a) concentration in males with IFG which is consistent with MetS, rather than diabetes, mediating CHD risk in men, in contrast to Turkish women in whom impaired glucose tolerance<sup>[8]</sup> and diabetes lead to the development of CHD<sup>[14]</sup>.

In the transition to MetS in men, beyond the development of atherogenic dyslipidemia and apoB, LDL and total cholesterol levels and the global risk profile are only marginally pronounced, regardless of the fasting glucose status. The transition to MetS in women, however, seems to be associated with increase in LDL-cholesterol, apoB, apoE, diastolic BP and little elevation in serum creatinine despite aging by 6 years. This may be attributed to the effect of apoB concentration substantially released from Lp (a) consequent to its aggregation to apoE/apoA-I in

**Table 3** Cox regression analysis for incident diabetes, by gender, in the whole sample and in subjects free of metabolic syndrome

	Whole sample						With MetS					
	Total, n = 238/2382 <sup>1</sup>		Men, n = 130/1140		Women, n = 108/1242		Total, n = 75/1360 <sup>1</sup>		Men, n = 49/687 <sup>1</sup>		Women, n = 26/673 <sup>1</sup>	
	HR	95%CI	HR	95%CI	HR	95%CI	HR	95%CI	HR	95%CI	HR	95%CI
Sex (female)	0.53	0.38;0.75					0.33	0.18-0.61				
Age (11 yr)	1.10	0.97-1.26	1.19	1.00-1.41	1.00	0.82-1.23	1.13	0.90-1.40	1.14	0.87-1.48	1.09	0.73-1.62
IFG <i>vs</i> normoglycemia	2.83	2.17-3.71	2.65	1.82-3.86	3.10	2.10-4.58	2.06	1.04-4.06	2.59	1.19-5.64	1.38	0.32-5.97
Body m. index (5 kg/m <sup>2</sup> )	1.74	1.53-1.98	1.91	1.55-2.34	1.64	1.39-1.94	1.77	1.34-2.33	1.86	1.24-2.78	1.70	1.18-2.46
HDL-cholesterol (12 mg/dL)	0.95	0.82-1.10	0.99	0.79-1.22	0.92	0.75-1.13	1.05	0.85-1.31	1.10	0.85-1.41	0.96	0.66-1.43
Statins usage	1.25	0.59-2.68	2.62	0.96-7.18	0.76	0.24-2.43	2.13	0.49-9.26	7.63	1.66-35.2	0	few users
Antihypertensive drugs	1.18	0.85-1.64	1.32	0.81-2.14	1.12	0.71-1.76	1.70	0.84-3.42	2.19	0.88-5.44	1.32	0.46-3.82
Current <i>vs</i> never smoking	1.04	0.73-1.47	1.11	0.72-1.71	0.86	0.47-1.59	0.67	0.37-1.23	0.85	0.42-1.73	0.20	0.03-1.48
Former <i>vs</i> never smoking	1.38	0.93-2.05	1.24	0.79-1.97	1.98	0.86-4.54	1.24	0.63-2.44	1.25	0.58-2.70	1.47	0.20-11.0

<sup>1</sup>Number of cases/number at risk. Mean age 52.5 years in the large sample. Missing people comprise lacking follow-up or missing values of individual factors in model. Impaired fasting glucose (IFG) was present in 17%, anti-hypertensive drug usage in 18% and current smoking in 34% in the whole sample at baseline. In the lower model, 400 men and 146 women were current smokers. Statins were used in 17 men and 37 women. HDL: High-density lipoprotein; MetS: Metabolic syndrome.

**Table 4** Cox regression analysis for incident coronary heart disease of impaired fasting glucose, by presence of metabolic syndrome and gender

	Whole sample						Without MetS						With MetS					
	Total, n = 359/2718 <sup>1,2</sup>		Men, n = 175/1324 <sup>2</sup>		Women, n = 184/1394 <sup>2</sup>		Total, n = 133/1569 <sup>1</sup>		Men, n = 74/795		Women, n = 59/774 <sup>1</sup>		Total, n = 226/1138 <sup>1</sup>		Men, n = 101/524		Women, n = 125/614	
	HR	95%CI	RR	95%CI	RR	95%CI	HR	95%CI	RR	95%CI	RR	95%CI	HR	95%CI	RR	95%CI	RR	95%CI
Female sex	0.94	0.72-1.22					0.82	0.54-1.27					0.98	0.70-1.38				
Age (11 yr)	1.49	1.34-1.66	1.52	1.31-1.76	1.46	1.26-1.71	1.71	1.46-2.02	1.71	1.37-2.13	1.75	1.37-2.24	1.33	1.15-1.52	1.40	1.14-1.73	1.24	1.02-1.51
IFG (IFG > 100 mg/dL)	0.93	0.71-1.21	0.71	0.46-1.10	1.10	0.78-1.55	0.37	0.14-0.998	0.47	0.15-1.53	0.22	0.03-1.64	0.96	0.72-1.29	0.75	0.46-1.23	1.10	0.76-1.61
Non-HDL chol. (35 mg/dL)	1.37	1.23-1.47	1.42	1.23-1.63	1.32	1.15-1.52	1.47	1.23-1.68	1.57	1.28-1.93	1.37	1.07-1.68	1.28	1.11-1.42	1.32	1.07-1.57	1.28	1.11-1.52
HDL cholesterol (12 mg/dL)	0.84	0.76-0.95	0.87	0.73-1.04	0.83	0.71-0.98	0.90	0.76-1.07	0.93	0.73-1.20	0.85	0.66-1.09	0.90	0.74-1.09	0.83	0.62-1.13	0.95	0.75-1.22
Systolic BP (25 mmHg)	1.42	1.28-1.56	1.72	1.49-2.04	1.31	1.16-1.49	1.35	1.13-1.60	1.60	1.22-2.15	1.22	0.95-1.52	1.35	1.19-1.56	1.72	1.38-2.15	1.22	1.03-1.42
Current <i>vs</i> never smoking	1.13	0.86-1.49	1.52	1.04-2.21	0.78	0.49-1.25	1.19	0.78-1.84	1.34	0.76-2.37	1.09	0.52-2.26	1.13	0.78-1.64	1.60	0.96-2.67	0.70	0.37-1.31
Former <i>vs</i> never smoking	1.13	0.79-1.60	1.23	0.81-1.88	1.42	0.66-3.05	1.05	0.58-1.90	0.93	0.45-1.92	2.22	0.90-6.22	1.15	0.74-1.79	1.38	0.81-2.33	0.95	0.30-3.03

<sup>1</sup>Number of cases/number at risk; <sup>2</sup>Participants with prevalent CHD and/or no follow-up were excluded. Impaired fasting glucose (IFG) was present in 17.7% of participants [8.5% of subjects without metabolic syndrome (MetS) and in 30.5% with MetS]. Current smoking in 35.8%, former smoking in 12.2% of participants. Statin drugs had an RR = 0.97 in 5 men without MetS and RR 1.06 in 9 men with MetS. RR 0.76 in all men. Statin drugs had an RR 1.96 in 8 women without MetS and RR 1.62 in 22 women with MetS. RR 1.69 in all women (P = 0.18). HDL: High-density lipoprotein; BP: Blood pressure.

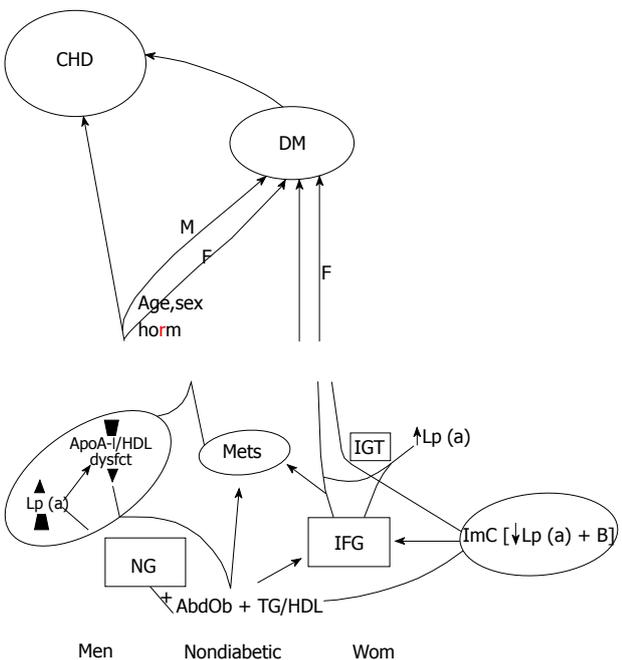
women with IFG, in whom a substantial decline of circulating Lp (a) (by 29%) seems to occur during a transition to MetS.

Beyond the augmentation of the three core components of MetS, two fundamental aspects in MetS that differ from non-MetS are compared with participants free of MetS: circulating Lp (a) is assayed to be lower by 7.5% (10.06 mg/dL *vs* 9.3 mg/dL); as a surrogate of immature pre-β HDL being more predominant in MetS in both sexes,

**Table 5 Clinical significance of impaired fasting glucose in regard to cardiometabolic risk depending on presence of metabolic syndrome**

Risk	Diabetes <sup>1</sup>	CHD <sup>2</sup>
Men without MetS	3-fold risk conferring	Protective
Men with MetS	3-fold risk conferring	Slightly protective
Women without MetS	Confers minimal risk	Protective
Women with MetS	3-fold risk conferring	Slightly risk conferring <sup>3</sup>

<sup>1</sup>Independent of age, body mass index, smoking, hypertension and high-density lipoprotein dysfunction; <sup>2</sup>Independent of age, smoking, systolic blood pressure non HDL cholesterol and dysfunctional HDL; <sup>3</sup>Likely related to autoimmune activation involving lipoprotein (a). MetS: Metabolic syndrome; CHD: Coronary heart disease.



**Figure 1 Schematic illustration.** Schematic illustration of major pathways derived from the current study leading from normoglycemia (NG)/impaired fasting glucose (IFG) to metabolic syndrome (MetS), diabetes (DM) and coronary heart disease (CHD) in non-diabetic men and women. Abdominal obesity and components of atherogenic dyslipidemia, the core of MetS, added to excess lipoprotein (Lp) (a), dysfunction of apoA-I and high-density lipoprotein (HDL) particles, commonly associated with normoglycemia rather than IFG, are major primary determinants of cardiometabolic risk, especially in men. Women often exhibit the added factor of lower assayable Lp (a) due to immune complex formation (ImC) which may lead from IFG via impaired glucose tolerance (IGT) to new-onset and established diabetes or via MetS to diabetes and, in turn, to CHD, contributed by menopause and alterations in sex hormones.

apoA-I/HDL-C ratio was higher (3.48 *vs* 3.01) in female MetS *vs* non-MetS, and (3.51 *vs* 3.15, respectively) in males. Each of these differences may partly account for the component of pro-inflammatory state in MetS.

We have pointed out that Turkish women are more prone to IGT than men due to a greater participation of chronic low-grade inflammation and HDL dysfunction<sup>[8]</sup>. The current study supports this notion and further provides evidence that autoimmune activation induced by Lp (a) may be the major determinant of IGT (and diabetes).

**Confirmation of HDL dysfunction and origin of low HDL cholesterol among Turks**

Current findings indicate further that relatively low levels of HDL cholesterol already occur in people free of MetS associated with a relatively wide waist and high triglycerides and that this trend is more pronounced in normoglycemic men and women. This observation is largely at variance with the proposition by Mahley *et al*<sup>[30]</sup> that low HDL cholesterol in Turkish adults has an isolated genetic origin irrespective of serum triglycerides.

**Hypothesis**

The presence of IFG in non-diabetic adults without MetS (8%-9%) indicates an atheroprotective risk factor profile since these people carry the “rudimentary phenotype” of abdominal obesity and atherogenic dyslipidemia (the crux of MetS) less. Males with IFG have, moreover, lower serum Lp (a) than normoglycemic men. Women generally possess substantially higher circulating Lp (a) than men and are subject to immune complex formation with loss of measurable Lp (a) (along with apoB) and concomitant increase in glycemia (yielding IFG) without elevated apoB-containing lipoproteins.

The slow process of established MetS develops as abdominal obesity and atherogenic dyslipidemia become pronounced and apoB-containing lipoproteins rise, during which modest loss of Lp (a) protein alone takes place in normoglycemic men and women, and major loss of both Lp (a) protein and apoB emerges in women with IFG. IFG in women represents commonly IGT as well rendering, as is known, diabetes more readily (Figure 1).

IFG confers a 3-fold risk of diabetes in each sex and the risk profile in IFG associated with MetS reduces the atheroprotective milieu in both sexes, especially in women due to the contributing effect of autoimmune activation. The limited immune complex formation in normoglycemic men requires merely the disappearance of atheroprotective properties of apoA-I (attained by effect of HtgW) to induce diabetes, contrasted to normoglycemic women who tend to hyperinsulinemia and need additional factors (changes in serum SHBG and testosterone) related to aging by several years. HDL dysfunction independent of IFG is an important contributor to the risk of CHD in MetS (as notable by HRs near unity).

Present findings support a concept that MetS is preceded over a long period by persistent minimal autoimmune activation manifesting as partly lower circulating assayable Lp (a) along with apoA-I dysfunctionality. This process represents increased insulin resistance despite normoglycemic levels prevailing.

A clinical implication of our IFG-related finding is to determine in a given individual whether IFG is or is not part of MetS (Table 5). If not, no further action may be needed other than assessing global cardiometabolic risk and undertaking preventive measures against diabetes in men. In subjects, especially women, with IFG associated with MetS (or hypertriglyceridemic waist phenotype), an oral GTT is mandatory to ascertain whether the clinician

is faced with IGT which should be regarded as nearly a DM-equivalent accompanied by autoimmune activation and HDL dysfunction. Standard efforts to reduce the pro-inflammatory state need to then be instituted, including insulin-sensitizing agents.

The lack of performance of a standard oral glucose tolerance test in this study evidently limits the important differentiation between the two types of pre-diabetic status, IFG and IGT, the absence of which, however, does not enhance and would rather tend to attenuate, the main finding of IFG representing a cardioprotective element in subjects free of MetS.

IFG status in non-diabetic people without MetS is wrought with excess diabetes risk, mainly due to a commonly underlying IGT, but is otherwise associated with a less adverse cardiovascular risk profile and reduced future CHD risk. "Protection" against CHD risk disappears as currently-defined MetS becomes established. Underlying the slowly progressive pro-inflammatory state is likely to be autoimmune activation linked to serum Lp (a).

## COMMENTS

### Background

Impaired fasting glucose (IFG), a common pre-diabetic state in middle-aged adults, often leads to diabetes but whether and to what extent it confers risk of coronary heart disease is controversial. A modest and non-linear association with risk of vascular disease was found in a meta-analysis.

### Research frontiers

Gender and presence of metabolic syndrome modulate cardiometabolic risk and might modulate IFG status as well. The authors stratified baseline characteristics distinguishing IFG from normoglycemia and future cardiometabolic risk by stratifying to gender and metabolic syndrome in this study. They showed that, surprisingly, normoglycemic subjects were distinct by a phenotype consisting of a significantly higher waist circumference, fasting triglyceride and lower high-density lipoprotein-cholesterol ("hypertriglyceridemic waist"), regardless of gender and metabolic syndrome (MetS). And notably, lipoprotein(a) tended to be lower in (particularly female) participants with MetS.

### Innovations and breakthroughs

This is the first study to report that IFG status in non-diabetic people without metabolic syndrome is independently associated not only with a less adverse cardiovascular risk profile than in normoglycemia, but also with a reduced future coronary heart disease risk. Only in men but not women without metabolic syndrome does IFG independently predict diabetes and the relative "protection" IFG affords against coronary heart disease disappears fully with the development of metabolic syndrome ("hypertriglyceridemic waist") only in women. The authors hypothesize autoimmune activation linked to serum lipoprotein(a) as responsible for the modulating phenomenon, which they found to be independently and apparently "inversely" related to components of insulin resistance in women.

### Applications

This knowledge may be utilized in population screening and more precise undertaking of preventive measures against diabetes and coronary heart disease, as well as in assessment of individual cardiometabolic risk. The hypothesis put forward also opens new avenues of research in the area of pathogenesis of new-onset diabetes and coronary heart disease.

### Peer review

The authors examined whether and to what extent IFG confers risk of diabetes or coronary heart disease by separately stratifying to gender and the presence of metabolic syndrome. The study disclosed that both factors modulated future risk. In women without metabolic syndrome, IFG was not associated with any cardiometabolic risk and in men it imparted only risk of diabetes. Coronary heart disease risk appeared to be dependent on the "hypertriglyceridemic waist" phe-

notype of the metabolic syndrome, especially in the female. The results suggest autoimmune activation involving lipoprotein(a) may be highly relevant in the pathogenesis of new-onset type 2 diabetes, with implications in both risk assessment and prevention of cardiometabolic risk, and open new avenues for research.

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## High adiponectin levels fail to protect against the risk of hypertension and, in women, against coronary disease: involvement in autoimmunity?

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### Abstract

**AIM:** To investigate whether serum adiponectin protects against cardiometabolic risk in a population sample with prevailing metabolic syndrome.

**METHODS:** Middle-aged adults representative of a general population with baseline circulating adiponectin measurements ( $n = 1224$ ) were analyzed prospectively at a mean of 3.8 years' follow-up, using continuous values or sex-specific tertiles. Total adiponectin was assayed by an ELISA kit. Type-2 diabetes was identified by criteria of the American Diabetes Association. Hypertension was defined as a blood pressure  $\geq$

140 mmHg and/or  $\geq 90$  mmHg and/or use of antihypertensive medication. Outcomes were predicted using Cox proportional hazards regression analysis in models that were controlled for potential confounders.

**RESULTS:** In models of multiple linear regression, sex hormone-binding globulin, fasting insulin (inverse) and, in men, age were significant independent covariates of serum adiponectin which further tended in women to be positively associated with serum creatinine. Cox regression analyses for incident coronary heart disease (CHD), adjusted for sex, age, non-HDL cholesterol, waist circumference and C-reactive protein, revealed significant inverse association with adiponectin tertiles in men but not women (HR = 0.66; 95%CI: 0.32-1.38 for highest tertile). Cox regression for type-2 diabetes in a similar model (wherein glucose replaced non-HDL cholesterol), adiponectin tertiles appeared to protect in each gender. HR for incident hypertension roughly displayed unity in each of the adiponectin tertiles ( $P$ -trend = 0.67).

**CONCLUSION:** High adiponectin levels failed to protect against the development of hypertension and, in women, against CHD, presumably paralleling impairment in renal function as well. Involvement of adiponectin in autoimmune complex with loss of antioxidative-antiatherogenic properties may be underlying.

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**Key words:** Adiponectin; Antioxidative function; Coronary heart disease; Creatinine; Type-2 diabetes; Hypertension

**Core tip:** The issue of whether serum adiponectin protects against cardiometabolic risk in people prone to metabolic syndrome was studied in 1224 Turkish adults at a mean of 3.8 years' follow-up. High adiponectin levels were surprisingly positively associated independently with serum creatinine in women and

further failed to protect against the development of hypertension in both sexes. In multivariable adjusted Cox proportional hazards regression analyses, protection against type-2 diabetes was apparent, but women were not protected against incident coronary heart disease by high serum adiponectin. Involvement of circulating adiponectin in autoimmune complex with loss of mainly antioxidative properties may be underlying.

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## INTRODUCTION

Serum adiponectin has been recognized in the past decade to be in an inverse relationship with hypertension<sup>[1]</sup> and low circulating adiponectin is equally recognized as a risk factor for hypertension, independent of its effects on insulin resistance and diabetes mellitus<sup>[2]</sup>. Plasma adiponectin levels correlated inversely more strongly with insulin levels and insulin resistance than the degree of obesity<sup>[3]</sup>. Higher adiponectin concentrations in diabetic men had reduced odds for renal dysfunction compared with the lowest adiponectin quartile<sup>[4]</sup>. These observations and numerous experimental studies<sup>[5]</sup> have shown that serum adiponectin exerts protective functions against cardiometabolic disorders, including hypertension, *via* insulin-sensitizing, anti-inflammatory and antiatherogenic actions.

Yet, marked elevations of plasma adiponectin levels have been reported in chronic kidney disease (CKD). In view of such an inverse relationship with renal function, the opinion has been expressed that the cardioprotective role of adiponectin in patients with CKD remains controversial<sup>[5]</sup>. Adiponectin levels were shown to be inversely associated with the glomerular filtration rate<sup>[6,7]</sup> which still needs a satisfactory explanation.

We have previously reported in Turkish adults that adiponectin levels were not only inconsistently related to excess adiposity, but also provided epidemiological evidence that serum adiponectin was markedly attenuated in its anti-inflammatory activities in women<sup>[8]</sup>. Moreover, in a cross-sectional analysis, serum adiponectin was not associated with diabetes and hypertension in men<sup>[9]</sup>.

In order to evaluate further the questionable protection by adiponectin against cardiometabolic disorders, we designed a prospective study after an intermediate follow-up of our original study sample wherein cross-sectional associations of adiponectin were also evaluated. Such a study might shed light on the determinants of attenuated activities of adiponectin and might also explain partly why a cardioprotective role of adiponectin is lacking in patients with CKD.

## MATERIALS AND METHODS

### Population sample

This study sample was recruited from the 2005/06 follow-up survey of the longitudinal Turkish Adult Risk Factor Study (TARF), a representative sample of adults in Turkey, the sampling details of which were described previously<sup>[9,10]</sup>. The study was approved by the Ethics Committee of the Istanbul University Medical Faculty. Written informed consent for participation was obtained. Partial logistical support was provided by the Turkish Ministry of Health. Data were obtained by history of the past years via a questionnaire, physical examination of the cardiovascular system, sampling of blood and recording of a resting 12 lead electrocardiogram. Serum concentrations of adiponectin were assayed among randomly selected fasting participants in a total of 561 men and 663 women, aged 37-79 years.

### Measurement of risk factors

Blood pressure (BP) was measured with an aneroid sphygmomanometer (Erka, Bad Tölz, Germany) in the sitting position on the right arm, and the mean of two recordings 3 minutes apart was recorded. Waist circumference was measured at the level midway between the lower rib margin and the iliac crest. Body mass index was calculated as weight divided by height squared ( $\text{kg}/\text{m}^2$ ). Cigarette smoking status was categorized into never, former and current smokers.

Blood samples were collected, spun at 1000 g, shipped to Istanbul and stored in deep-freeze at  $-75\text{ }^\circ\text{C}$  until analyzed. Serum concentrations of hsC-reactive protein (CRP), apolipoprotein (apo) B, apo A-I, complement C3 and lipoprotein (Lp)(a) were measured by nephelometry (BN Prospec, Behring Diagnostics, Westwood, MA). Serum concentration of total adiponectin was assayed by a sandwich enzyme-linked immunosorbent assay system (Adiponectin ELISA BioVendor, BioVendor Lab. Medicine, Inc, Czech Republic) at a central laboratory. Serum concentrations of total cholesterol, fasting triglycerides, glucose, HDL cholesterol (HDL-C, directly) and low-density lipoprotein cholesterol (directly) were determined by using enzymatic kits from Roche Diagnostics (Mannheim, Germany) with a Hitachi 902 autoanalyzer. Serum concentrations of sex hormone-binding globulin (SHBG), insulin and thyroid stimulating hormone (TSH) were measured by the electrochemiluminescence immunoassay ECLIA on Roche Elecsys 2010 using Roche kits (Roche Diagnostics, Mannheim, Germany).

### Definitions

Hypertension was defined as a blood pressure  $\geq 140$  mmHg and/or  $\geq 90$  mmHg and/or use of antihypertensive medication. Type-2 diabetes was diagnosed with the criteria of the American Diabetes Association<sup>[11]</sup>, namely by self report or when plasma fasting glucose was  $\geq 7$  mmol/L or when 2-h postprandial glucose was

**Table 1** Characteristics of sample (*n* = 1224) by gender and serum adiponectin (in  $\mu\text{g/mL}$ ) tertiles

	<i>n</i>	Men ( <i>n</i> = 561)							Women ( <i>n</i> = 663)																				
		1		4.94 <sup>1</sup>		2		8.50		3		15.36		Anova		1		6.12 <sup>1</sup>		2		10.87		3		19.67		Anova	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	<i>P</i> -trend	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	<i>P</i> -trend		
Age (yr)	1224	52.2	10.1	53.8	11	58.1 <sup>4</sup>	11.9	< 0.001	52.8	10.3	53.5	11.9	57.1 <sup>4</sup>	12	< 0.001														
BMI (kg/m <sup>2</sup> )	1213	28.2	4	29.3	4.3	27.3	4.5	0.23	31.8 <sup>4</sup>	6	30.3	4.8	30.1	5.7	0.003														
Waist circumfer. (cm)	1217	97.3	11.1	96.8	10.6	95	11.9	0.11	94.3	12.7	92.1	11.4	91.2	12.7	0.024														
Systolic BP (mmHg)	1217	120.6	18.8	121.3	19.6	121.8	20.3	0.83	130.5	23.9	126.4	21.7	127.3	23.7	0.15														
Diastolic BP (mmHg)	1217	77.8	10.8	77.7	10.3	77.6	10.6	0.98	80.8	11.5	79.7	10.4	80	11.8	0.54														
Complement C3 (g/L)	610	1.36	0.27	1.29	0.27	1.23	0.27	0.007	1.44	0.29	1.37	0.33	1.24	0.24	< 0.001														
CRP <sup>3</sup> (mg/L)	1143	2.26	1.25-5.06	2.11	1.02-3.9	1.42 <sup>4</sup>	0.76-3.2	< 0.001	2.72	1.15-6.33	2.61	1.21-6.18	2.29	1.08-4.0	0.025														
Total cholest. (mg/dL)	1213	191.3	40	194.8	38	190	40.3	0.46	201	41.7	205.4	53	204.9	44.3	0.54														
Fast. glucose (mg/dL)	1124	99	39	99.6	37.8	94	25.8	0.27	101.9	43.9	99	38.2	93.7	32	0.093														
ApoB (mg/dL)	1116	107.6	26.7	105.3	28.9	100.4	46.9	0.15	106	26.3	104.3	32.5	106.9	37.5	0.72														
Creatinine (mg/dL)	1144	1.02	0.19	1.02	0.22	0.99	0.22	0.24	0.78	0.21	0.79	0.18	0.84	0.42	0.066														
Fast. triglycer. <sup>3</sup> (mg/dL)	1123	160.8 <sup>4</sup>	108-227	138.1	100-187	126.2	89-174	< 0.001	146.2	108-206	133.5	99.9-182	117 <sup>4</sup>	83.5-164	< 0.001														
HDL-cholest. (mg/dL)	1207	35.6	8.3	39.5	9.8	43 <sup>4</sup>	11.3	< 0.001	43.9	11.5	45.6	10.6	49.4 <sup>4</sup>	11.7	< 0.001														
Apo A-I (mg/dL)	1103	129.7	25	134.7	25.1	138.9	25.8	0.004	144.3	26.4	145.7	29.9	152.6	28.1	0.008														
Lp (a) <sup>3</sup> (mg/dL)	764	8.16	3.8-18.2	7.93	3.2-19.4	9.36	3.4-20.6	0.52	12.1	5.3-24.5	13.3	5.7-23.6	12.4	4-29.4	0.85														
Thyroid SH <sup>3</sup> (mIU/L)	532	1.06	0.7-1.6	1.05	0.6-1.7	1.01	0.67-1.5	0.89	1.53	0.97-2.45	1.47	0.84-2.3	1.37	1.0-2.46	0.70														
Current/former smok (%)	1218	58.4	20	47.9	21.1	45.9	29	0.035	16	3.7	18.6	3.6	14.7	3.7	0.87														
Prevalent diabetes <sup>2</sup> (%)	1219	11.8		12.6		13.1		0.93	17.7		12.1		12.9		0.18														
Incident CHD, <i>n</i> (%)	1061	13 (8.2)		11 (6.7)		4 (2.5)		0.086	21 (11.1)		8; 4		16 (8.4)		0.029														

<sup>1</sup>Geometric mean adiponectin value of the tertile; <sup>2</sup>Excluded from the study; <sup>3</sup>Median and interquartile range; <sup>4</sup>Values differing from both of the other tertiles. CRP: C-reactive protein; BMI: Body mass index; BP: Blood pressure; CHD: Coronary heart disease; HDL: High-density lipoprotein; LP: Lipoprotein.

> 11 mmol/L. MetS was identified when 3 out of the 5 criteria of the National Cholesterol Education Program ATP-III were met, modified for prediabetes (fasting glucose 5.56-6.95 mmol/L)<sup>[12]</sup> and further for male abdominal obesity using as cut-point  $\geq 95$  cm, as assessed in the TARF study<sup>[13]</sup>.

Information on the mode of death was obtained from first-degree relatives and/or health personnel of the local health office. Cause of death was assigned with the consideration also of pre-existing clinical and laboratory findings elicited during biennial surveys. CHD death comprised of death from heart failure of coronary origin and fatal coronary event. Nonfatal CHD was identified by the presence of angina pectoris, a history of myocardial infarction with or without accompanying Minnesota codes of the ECG<sup>[14]</sup> or a history of myocardial revascularization. Typical angina and, in women, age > 45 years were prerequisite for a diagnosis when angina was isolated. ECG changes of "ischemic type" of greater than minor degree (codes 1.1-2, 4.1-2, 5.1-2, 7.1) were considered as myocardial infarct sequelae or myocardial ischemia, respectively.

### Statistical analysis

Descriptive parameters were shown as mean  $\pm$  standard deviation or in percentages. Distribution in variables with skewed distribution [total adiponectin, CRP, SHBG, insulin, lipoprotein (a) and TSH] was shown in median and interquartile range and log-transformed analyses were used. ANOVA *P*-trend analyses and pairwise comparisons with post hoc Tukey HSD were made to detect significance between groups; two-sided *t*-tests and Pearson's chi-square tests were used to analyze the differences between means and proportions of other groups.

Multiple linear regression analyses were performed with continuous parameters related to inflammation. To detect nonlinearity of associations with outcome, tertiles of adiponectin (6.9-11.7 in men and 8.8-14.9 women  $\mu\text{g/mL}$  formed the intermediate tertiles) were assessed. Cox proportional hazard regression models were used for incident cases of CHD, diabetes and hypertension after exclusion of prevalent cases, at a mean follow-up of 3.82 years. HR estimates and 95%CI were obtained in models that adjusted for sex, age and relevant confounders, expressed in terms of 1-SD increment. A value of *P* < 0.05 on the two-tail test was considered statistically significant. Statistical analyses were performed using SPSS-10 for Windows.

## RESULTS

Geometric mean total adiponectin values in women (10.9  $\mu\text{g/mL}$ ) were higher by 27% than in men (8.6  $\mu\text{g/mL}$ , *P* < 0.001). MetS was identified in 46% of individuals at baseline (9). Mean follow-up period constituted 3.82  $\pm$  1.47 years (range 2 to 6 years) which yielded a total follow-up 3820 person-years for incident diabetes; 3340 person-years for incident CHD, after exclusion of prevalent cases at baseline (160 and 150 cases, respectively) and participants lost to follow-up (64 and 199 cases, respectively). Fifty-two new cases of diabetes (12.6 per 1000 person-years) and 73 of CHD (17.6 per 1000 person-years) developed during the follow-up.

Characteristics of the sample population are presented in Table 1, separately for men and women and stratified by serum adiponectin tertiles.

When correlations between log-transformed Lp (a),

**Table 2** Linear regression analysis for serum adiponectin

	Total		Men		Women			Total		Men		Women	
	$\beta$ coeff.	P value	$\beta$ coeff.	P value	$\beta$ coeff.	P value		$\beta$ coeff.	P value	$\beta$ coeff.	P value	$\beta$ coeff.	P value
Model 1	n = 513		n = 252		n = 261		Model 2	n = 454		n = 223		n = 231	
Sex (female)	1.20	< 0.001					Sex (female)	1.285	< 0.001				
Age (11 yr)	1.05	0.055	1.10	0.005	0.997	0.93	Age (11 yr)	1.04	0.13	1.067	0.079	1.007	0.88
Fasting insulin <sup>1</sup>	0.79	0.009	0.80	0.016	0.79	0.033	C-reactive protein <sup>1</sup>	0.90	0.057	0.83	0.011	0.98	0.80
SHBG1 (nmol/L)	2.14	< 0.001	1.76	0.001	2.02	< 0.001	SHBG1	1.97	< 0.001	1.77	0.002	2.08	< 0.001
Creatinine (0.25 mg/dL)	1.04	0.67	0.992	0.82	1.085	0.052	Fasting insulin <sup>1</sup>	0.84	0.026	0.83	0.06	0.86	0.20
							Creatinine (0.25 mg/dL)	1.04	0.14	1.01	0.78	1.081	0.086
							Waist circumference (12 cm)	1.023	0.41	1.021	0.57	1.025	0.52
							Lipoprotein(a) <sup>1</sup>	1.003	0.94	1.07	0.38	0.96	0.60

All models were significant ( $P < 0.001$ ), explained 16%/17% of adiponectin variance in genders combined, 14%/16% in men and 12% in women. <sup>1</sup>Log-transformed values. SHBG: Sex hormone-binding globulin.

creatinine and apoB were examined separately in sex-specific adiponectin tertiles, in men, Lp (a) was significantly correlated with apo B (0.20,  $P = 0.035$ ) in the mid-tertile and tended to be so with creatinine (0.16,  $P = 0.089$ ) in the lowest adiponectin tertile. In women, Lp (a) and creatinine were not correlated in the adiponectin top tertile and were inversely correlated (-0.24,  $P = 0.004$ ) in the mid-tertile.

Table 2 shows findings of a multiple linear regression analysis for baseline covariates of circulating adiponectin in two significant models. In Model 1, apart from the female sex, levels of SHBG (positively) and insulin (inversely) were associated in each gender, while creatinine was positively associated in women with adiponectin at borderline significance. When waist circumference, CRP and Lp (a) were further added in Model 2, CRP emerged a further inverse covariate in men, beyond the persisting female sex, circulating SHBG and insulin as significant and, in women, creatinine as borderline significant covariates.

Table 3 demonstrates results of multivariable Cox proportional hazard regression analyses of adiponectin tertiles for the development of CHD, diabetes and hypertension, separately by gender. With respect to type-2 diabetes, fasting glucose and waist girth were significant predictors in each sex and the highest adiponectin tertile was a significantly inverse predictor in the total sample.

CHD risk was predicted in a multivariable Cox model adjusted also for CRP by non-HDL-cholesterol only at borderline significance in men and waist girth in the whole sample, while HRs in the higher two tertiles of adiponectin revealed significant inverse associations (Table 3). In women in contrast, an inverted J-shaped risk curve was apparent inasmuch as RR in the highest tertile did not reach significance, whereas in the mid-tertile HR seemed to be in protective direction more than anticipated.

Cox model for incident hypertension comprising sex, age, waist girth, CRP and adiponectin tertiles disclosed female sex (HR = 1.63), age (HR = 1.62) and waist circumference to be significant predictors. Adiponectin tertiles were not significantly associated in either sex, and slightly tended in men to be associated with elevated risk

of hypertension.

## DISCUSSION

In this prospective population-based study in middle-aged adults, we extended our previously reported evidence<sup>18,91</sup> for impaired anti-inflammatory/antioxidative and atheroprotective properties of high serum adiponectin levels, insofar as the mid and highest tertiles were not protective against risk of hypertension in both sexes and the highest tertile not against risk of CHD in women. Serum adiponectin in women, at variance from that in men, tended to be independently, positively and linearly associated with creatinine concentrations. Collectively, the provided evidence suggested that an autoimmune process involving adiponectin may operate, rendering the inability to protect against hypertension and, in women, against CHD, as well as in contributing to renal functional impairment. These findings diverge in part from those previously reported; details and possible reasons are discussed below.

### Risk of hypertension and CHD

An independent inverse relationship between adiponectin and hypertension<sup>1,2,15-17</sup> or blood pressure has been repeatedly demonstrated. In the prospective case-control study on South Chinese adults, diabetic patients were excluded<sup>17</sup>. The action of adiponectin is believed to be due to protection against endothelial dysfunction mediated by AMP-activated protein kinase-eNOS signaling and COX-2-prostaglandin I<sub>2</sub> signaling pathways, changes in macrophage function and up-regulation by renin-angiotensin system inhibition<sup>18</sup>. Nonetheless, BP was not found to be related to plasma adiponectin levels in 180 overweight and obese Asian subjects<sup>19</sup>. In the current study, prospective analysis of the development of hypertension in 126 subjects among 661 non-hypertensive men and women at baseline showed a lack in protective function of the intermediate and high adiponectin tertiles in either sex, independent of waist girth and CRP concentrations. Relative risks were even above unity. This may be attributed to alterations of the adipocytokine secondary to involvement in autoimmune activation

**Table 3** Cox regression analyses of serum adiponectin tertiles for incident diabetes, coronary heart disease and hypertension, adjusted for sex, age and relevant confounders

	Total HR	95%CI	Men HR	95%CI	Women HR	95%CI
Diabetes		40/761 <sup>2</sup>		21/333 <sup>2</sup>		19/428 <sup>2</sup>
Adiponectin mid-tertile	0.64	0.32-1.31	0.83	0.30-2.28	0.35	0.11-1.09
Adiponectin top-tertile	0.26	0.10-0.69	0.28	0.07-1.17	0.23	0.06-0.88
Fasting glucose (25 mg/dL)	1.60	1.22-2.04	1.49	1.08-2.09	2.25	1.35-3.72
Waist circumference (12 cm)	1.88	1.43-2.46	2.04	1.44-2.88	1.78	1.13-2.78
Creatinine (0.25 mg/dL)	1.08	0.74-1.58	0.77	0.37-1.60	1.18	0.87-1.60
C-reactive protein <sup>1</sup> , 3-fold	1.21	0.97-1.52	1.10	0.80-1.51	1.36	0.96-1.73
Coronary disease		66/805 <sup>2</sup>		25/358 <sup>2</sup>		41/447 <sup>2</sup>
Adiponectin mid-tertile	0.54	0.30-0.97	0.8	0.34-1.92	0.39	0.17-0.90
Adiponectin top-tertile	0.49	0.26-0.91	0.31	0.09-1.05	0.66	0.32-1.38
Non-HDL cholesterol. (35 mg/dL)	1.07	0.87-1.28	1.37	0.97-1.93	0.93	0.70-1.19
Waist circumference (12 cm)	1.46	1.18-1.82	1.28	0.89-1.84	1.60	1.22-2.08
Creatinine (0.25 mg/dL)	1.20	0.91-1.58	1.36	0.85-2.17	1.05	0.69-1.60
C-reactive protein <sup>1</sup> , (3-fold)	1.12	0.95-1.32	1.06	0.81-1.39	1.18	0.95-1.47
Hypertension		120/541 <sup>2</sup>		53/274 <sup>2</sup>		67/267 <sup>2</sup>
Adiponectin mid-tertile	1.08	0.71-1.91	1.23	0.62-2.43	1.03	0.56-1.89
Adiponectin top-tertile	0.77	0.55-1.59	1.08	0.51-2.30	0.64	0.33-1.24
Waist circumference (12 cm)	1.41	1.14-1.74	1.28	0.93-1.76	1.53	1.14-2.06
Creatinine (0.25 mg/dL)	1.06	0.82-1.37	1.15	0.80-1.66	1.08	0.74-1.59
C-reactive. protein <sup>1</sup> , 3-fold	0.96	0.85-1.09	1.08	0.90-1.30	0.90	0.76-1.06

<sup>1</sup>Log-transformed. All models were additionally sex- and age-adjusted. Referent low adiponectin tertile (< 6.9 men and < 8.8 µg/mL women). <sup>2</sup>Number of incident cases/number at risk. Mean creatinine values at baseline were 0.994 in men and 0.776 mg/dL in women, and ages 53.5 and 53 years, respectively.

(as outlined below).

In regard to the atheroprotective property of adiponectin, cohort studies have yielded conflicting results. The large Rancho Bernardo study<sup>[20]</sup> reported divergent associations between serum adiponectin levels and combined prevalent and incident CHD and mortality, in contrast to the German cross-sectional case-control study overwhelmingly on males<sup>[21]</sup> which reported lower multi-adjusted odds ratios in increasing adiponectin quintiles. In essential agreement with the findings of Lawlor *et al*<sup>[22]</sup> who reported a lack of prediction of CHD by adiponectin in women, we found that the highest adiponectin tertile in women appeared not to protect against the CHD risk, despite an apparent significant protection in men. The interesting gender difference is consistent with the notion of loss of antioxidative properties mainly in postmenopausal women who exhibit a reduced concentration of SHBG, a major determinant of adiponectin<sup>[8]</sup>, and a notable positive association with serum creatinine.

#### Sex-specific positive association with serum creatinine

Our linear regression models for baseline adiponectin concentrations demonstrated similar associations across sexes with respect to SHBG and insulin levels, but diverged regarding serum creatinine. Inverse associations (as noted in men) are anticipated between circulating adiponectin and creatinine which emerged to be positive in women, albeit at  $P = 0.052$ . This is consistent with a setting in which high adiponectin levels in a subset of the female sample were converted pro-oxidative to mediate endothelial dysfunction, acquiring attenuated atheroprotective effects, concomitantly with a reduced glomerular filtration rate. In view of our recent reports of higher CHD risk in women in the bottom creatinine quartile

compared with the two intermediate quartiles<sup>[23,24]</sup>, immune complex formation with adiponectin may be suggested.

#### Diabetes risk

In regard to the risk of type-2 diabetes, circulating adiponectin seemed to exert a protective effect. This is in line with previous reports on low adiponectin levels and diabetes risk<sup>[16,25-27]</sup>. A multi-SNP genotypic risk score tested in nearly 40000 individuals was positively associated with the risk of type-2 diabetes<sup>[28]</sup>. A protective effect found against diabetes parallels our finding of a significant linear and inverse association of adiponectin with fasting insulin. Current findings highlight that the insulin-sensitizing property of adiponectin may be retained while anti-oxidative and macrophage properties related to protection against hypertension and CHD may be attenuated.

#### Autoimmune activation in mechanistic explanation of findings

Adiponectin may well function in women with a pro-inflammatory state as an immune component, directed presumably against oxidized creatinine, may assume pro-inflammatory properties and induce impairment in endothelial and renal function, independent of low circulating SHBG and hyperinsulinemia. This view is supported by the highest compared with the intermediate adiponectin tertile not significantly protecting against CHD risk. The involvement of adiponectin in immune activation in women may result both in endothelial dysfunction-mediated renal dysfunction (CRP elevation) and failure to protect against CHD risk. This may explain the concomitantly raised risk of myocardial infarction and

all-cause mortality observed in patients with decreasing renal function<sup>[29]</sup> and is consistent with our hypothesized mechanism<sup>[30]</sup>.

That the described lack of association in women between the high adiponectin tertile and CHD risk was not related to potential inadequate statistical power of the sample is negated by the mid-tertile displaying a significant inverse RR, coupled to the observation that the power of the top tertile in men did disclose a significant inverse RR. Thus, the lack of protection against CHD risk appears a valid gender-specific phenomenon.

### Accounting for the lack of useful prognostic value of adiponectin in renal failure

Studies demonstrated that adiponectin concentrations were paradoxically inversely associated with glomerular filtration rates<sup>[6,7]</sup> and that in advanced kidney disease patients, cardiovascular and all-cause mortality was raised with increasing adiponectin levels<sup>[31]</sup>. Our relevant finding, together with these observations, indicates that circulating adiponectin and creatinine may parallel each other under conditions of a pro-inflammatory state. In studying the relationship of plasma adiponectin with inflammatory biomarkers and metabolic status in 180 patients with mild to moderate CKD, Norata and co-workers emphasized that, given that adiponectin synthesis is not increased and excretion not impaired, the reason for the increased adiponectin level was still unclear<sup>[32]</sup>. Our proposed hypothesis of adiponectin involvement in autoimmune activation can explain these phenomena hitherto unaccounted for.

### Limitations and strengths

The comparatively brief follow-up limited the outcomes sought in a substantial proportion of the study sample, limiting the statistical power in fully assuring of not dealing with a chance finding in certain analyses, yet still did not preclude the emergence of significant findings in the opposite sex or the other adiponectin tertile. Residual confounding may not be completely excluded. We did not document the postulated hypothesis by immunoassays, if this is ever possible; however, both present findings and those previously reported support each other in this direction. The large, population-based study sample exhibiting a relatively high prevalence of enhanced low-grade inflammation forms strength, while possibly partly limiting applicability of findings to some other ethnic populations at large. Availability of measurements of diverse relevant variables that are not commonly studied in previous reports on adiponectin forms a further strength of the study.

In conclusion, added to our previous report of impaired protective properties of high circulating adiponectin in middle-aged Turkish adults, elevated levels were found to be not protective against the risk of hypertension in both genders and in women against CHD risk. At variance from men, serum adiponectin in women tended to be independently and positively associated with cre-

atinine concentrations. We propose that involvement of adiponectin in autoimmune activation may underlie both the lack of stated protection and a concomitant presumable contribution to renal functional impairment.

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## COMMENTS

### Background

Serum adiponectin exerts protective functions against cardiometabolic disorders, including hypertension, *via* insulin-sensitizing, anti-inflammatory and antiatherogenic properties. Yet, adiponectin levels are often inversely associated with glomerular filtration rate which still needs a satisfactory explanation.

### Research frontiers

Gender and the presence of metabolic syndrome/pro-inflammatory state modulate cardiometabolic risk and might modulate the protective function of serum adiponectin. The authors analyzed baseline covariates of adiponectin and found that serum creatinine was, surprisingly, a positive independent covariate. In prospective analyses, although incident type-2 diabetes appeared to be protected by the high adiponectin tertile, no evidence of protection was elicited against incident hypertension and, in women, against coronary heart disease. These findings suggested impairment of some properties of serum adiponectin due to enhanced low-grade inflammation and autoimmune activation.

### Innovations and breakthroughs

This study offers a potential explanation to the controversies on the role of serum adiponectin in chronic kidney disease, but shows also that part of the properties (antioxidative) of this cytokine may become impaired, analogous to that recently documented regarding high-density lipoprotein, resulting in lack of protection against hypertension risk and, sex-specifically, against future coronary heart disease (CHD) risk. As responsible for the modulating phenomenon, the authors hypothesize that autoimmune activation in women is linked to serum creatinine and adiponectin to mediate renal dysfunction and the associated CHD risk.

### Applications

This knowledge may be utilized in population screening and more precise undertaking of preventive measures against diabetes and coronary heart disease, as well as in assessment of individual cardiometabolic risk. The hypothesis put forward also opens new avenues of research in the area of pathogenesis of chronic kidney disease and coronary heart disease.

### Peer review

The authors examined whether and to what extent circulating adiponectin protects against the risk of hypertension, diabetes or coronary heart disease, separately in each gender. The study revealed that serum adiponectin was positively and independently associated in women linearly with serum creatinine, a pro-inflammatory compound. Prospective multivariable analyses disclosed that the development of CHD risk was not reduced in women by the highest adiponectin tertile, nor was the incident hypertension risk in either gender. By showing on the other hand that adiponectin tertiles protected against incident type-2 diabetes, evidence was provided that insulin-sensitizing properties remained intact, as opposed to the loss of antioxidative and antiatherogenic action. The results suggest that the operation of autoimmune activation involving adiponectin and creatinine may contribute to the pathogenesis of elevated BP and CHD. This may carry implications in both risk assessment and prevention of cardiometabolic risk, warranting new avenues for research.

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## Sequential elevation of autoantibodies to thyroglobulin and glutamic acid decarboxylase in type 1 diabetes

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### Abstract

We have previously reported the high levels of glutamic acid decarboxylase 65 autoantibodies (GAD65A) in patients with type 1 diabetes and autoimmune thyroid disease. Here we describe a 32-year-old Japanese female with a thirteen-year history of type 1 diabetes whose levels of GAD65A were elevated just after the emergence of anti-thyroid autoimmunity. At 19 years of age, she developed diabetic ketoacidosis and was diagnosed with type 1 diabetes. She had GAD65A, insulinoma-associated antigen-2 autoantibodies (IA-

2A), and zinc transporter-8 autoantibodies (ZnT8A), but was negative for antibodies to thyroid peroxidase (TPOAb) and thyroglobulin (TGAb) at disease onset. ZnT8A and IA-2A turned negative 2-3 years after the onset, whereas GAD65A were persistently positive at lower level (approximately 40 U/mL). However, just after the emergence of TGAb at disease duration of 12.5 years, GAD65A levels were reelevated up to 5717 U/mL in the absence of ZnT8A and IA-2A. Her thyroid function was normal and TPOAb were consistently negative. She has a HLA-*DRB1\*03:01/\*04:01-DQB1\*02:01/\*03:02* genotype. Persistent positivity for GAD65A might be associated with increased risk to develop anti-thyroid autoimmunity.

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**Key words:** Autoimmune thyroid disease; Case report; Glutamic acid decarboxylase autoantibodies; Type 1 diabetes

**Core tip:** This paper describes a case of type 1 diabetes whose levels of glutamic acid decarboxylase 65 autoantibodies (GAD65A) were reelevated just after the emergence of anti-thyroid autoimmunity at disease duration of 12.5 years without any clinical signs of thyroid dysfunction. This case report suggests that persistent positivity for GAD65A is associated with increased risk to develop anti-thyroid autoimmunity.

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## INTRODUCTION

Type 1 diabetes is an autoimmune disease against pancreatic islet  $\beta$  cells and is often complicated with other autoimmune diseases, of which autoimmune thyroid disease (AITD) is the most frequent<sup>[1]</sup>. It has been reported that the prevalence of autoantibodies to thyroid peroxidase (TPOAb) and/or thyroglobulin (TGAb) are 15%-30% in patients with type 1 diabetes at the time of diagnosis of the diabetes<sup>[1-3]</sup>. Furthermore, there is heterogeneity in the natural history of AITD in patients with type 1 diabetes: AITD may be diagnosed either at diabetes onset or during the follow-up, and the measurement of TPOAb and TGAb is useful to predict the development of future AITD. We and others have previously reported the association between anti-thyroid autoimmunity and anti-islet autoantibodies, especially glutamic acid decarboxylase 65 autoantibodies (GAD65A) and zinc transporter 8 autoantibodies (ZnT8A)<sup>[4-7]</sup>. The levels and the prevalence of GAD65A are higher and more persistent in type 1 diabetic patients with AITD compared to type 1 diabetes alone<sup>[4]</sup>. Here we report a 32-year-old Japanese female with a thirteen-year history of type 1 diabetes whose levels of GAD65A were reelevated just after the emergence of anti-thyroid autoimmunity.

## CASE REPORT

A 32-year-old Japanese female had developed diabetic ketoacidosis at 19 years of age and diagnosed as type 1 diabetes. She was immediately started the intensive insulin therapy and referred to our hospital for glycemic control after one month of diabetes onset. Her past medical history was unremarkable and her family history was negative for diabetes or autoimmune diseases. Her body mass index was 19.4 kg/m<sup>2</sup> and blood pressure was 108/72 mmHg. Neither exophthalmos nor diabetic retinopathy was observed. Thyroid gland was slightly enlarged. There were no abnormal findings of the heart, lungs, or abdomen. Neurological examination was also normal. As shown in Table 1, endogenous insulin secretory capacity was decreased (Urine C-peptide 5.5  $\mu$ g/d) and GAD65A (4342 U/mL; normal range < 1.5 U/mL), IA-2A (index 0.769; normal range < 0.018) and ZnT8A (index 0.055; normal range < 0.007) were all positive. However, both TPOAb and TGAb were negative. She had HLA-DRB1\*03:01/\*04:01-DQB1\*02:01/\*03:02 genotype. She was treated by insulin aspart before each meal (30 U) and glargine at lunch and dinner (10 U) and her hemoglobin A1c (HbA1c) levels were maintained between 6.2% and 7.5%.

During the follow-up her GAD65A, anti-thyroid antibodies and thyroid function were monitored regularly. The GAD65A level gradually decreased and reached at 40 to 50 U/mL at > 10 years after diabetes onset. Her TPO/TGABs remained negative and thyroid function was within normal range. However, GAD65A were reelevated to 90 U/mL thirteen years after diabetes onset. Then we

**Table 1** Laboratory findings at the first visit

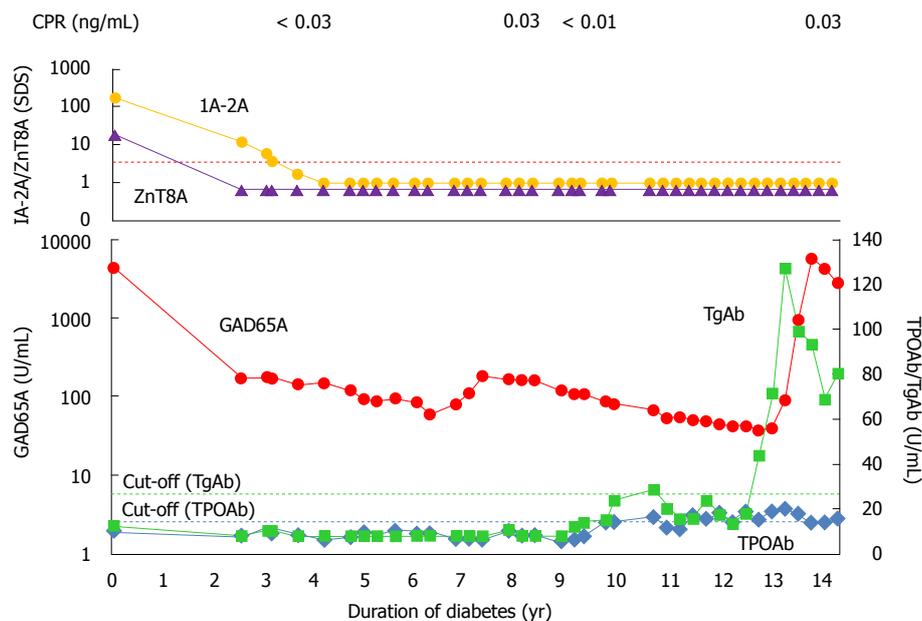
Laboratory findings	
Urinalysis	
Sugar	(-)
Protein	(-)
Ketone	(-)
Hematological data	
WBC (/ $\mu$ L)	$5.0 \times 10^3$
RBC (/ $\mu$ L)	$4.5 \times 10^6$
Hb (g/dL)	13.1
Hct (%)	39.9
Plt (/ $\mu$ L)	$21.7 \times 10^4$
Biochemical data	
TP (g/dL)	6.6
AST (IU/L)	11
ALT (IU/L)	11
$\gamma$ -GTP (IU/L)	10
TC (mg/dL)	184
TG (mg/dL)	50
Cr (mg/dL)	0.5
BUN (mg/dL)	14
PG (mg/dL)	100
HbA1c (%)	11.90
U-CPR ( $\mu$ g/d)	5.5
Immunological data	
GAD65A (U/mL)	4342
IA-2A (index)	0.769
ZnT8A (index)	0.055
TPOAb (U/mL)	(-)
TGAb (U/mL)	(-)
HLA-DRB1	*03:01/*04:01
HLA-DQB1	*02:01/*03:02

HbA1c: Hemoglobin A1c; ZnT8A: Zinc transporter-8 autoantibodies; TPOAb: Thyroid peroxidase antibody; TGAb: Thyroglobulin antibody; WBC: White blood cell; RBC: Red blood cell; TP: Total protein; AST: Aspartate transaminase; ALT: Alanine aminotransferase;  $\gamma$ -GTP:  $\gamma$ -glutamyl transpeptidase; TC: Total cholesterol; TG: Triglyceride; Cr: Creatinine; BUN: Blood urea nitrogen; PG: Plasma glucose.

measured anti-islet autoantibodies and anti-thyroid antibodies in her stored samples (Figure 1). ZnT8A and IA-2A turned negative 2-3 years after the onset, whereas GAD65A had been persistently positive at lower level. However, TGAb emerged at disease duration of 12.5 years and GAD65A levels were reelevated thereafter up to 5717 U/mL in the absence of ZnT8A and IA-2A. Ultrasound examination showed a thyroid gland of homogenous parenchyma with normal size. Furthermore, no significant elevation of TPOAb was observed and her thyroid function was normal throughout the clinical course. Her serum C-peptide level was undetectable at disease duration of 4 years and did not change after the emergence of anti-thyroid autoimmunity. Furthermore, her metabolic control and insulin requirement did not change (HbA1c 6.8%-7.1%, insulin requirement 35-41 U/d).

## DISCUSSION

There were many reports on the association between AITD and type 1 diabetes. However, little is known on the dynamics of the humoral autoimmunity to islet autoantigens in association with anti-thyroid autoimmunity in



**Figure 1** Time course of anti-islet autoantibodies and anti-thyroid antibodies. Dotted lines indicate the cut-off values of antibodies. The levels of IA-2A and ZnT8A were expressed as SDS. IA-2A: Insulinoma-associated antigen-2 autoantibodies; ZnT8A: Zinc transporter-8 autoantibodies; SDS: Standard deviation score; GAD: Glutamic acid decarboxylase.

type 1 diabetic patients who have no evidence of thyroid autoimmunity at disease onset. In patients with type 1 diabetes positive for glutamic acid decarboxylase autoantibodies (GADA), a higher prevalence of anti-thyroid antibodies was reported as compared to those without GADA<sup>[5,8]</sup>. Furthermore, we have previously reported that patients with type 1 diabetes and AITD (*i.e.*, autoimmune polyendocrine syndrome type 3; APS3) show the higher levels of GAD65A compared to patients with type 1 diabetes alone in both cross-sectional and longitudinal observations<sup>[4]</sup>. Because high levels of GADA are observed in insulin-deficient patients as same as that of our case, production of GADA may not be associated with the residual  $\beta$  cell antigens. Furthermore, it has been reported that GAD is not only expressed in  $\beta$  cells but also in the thyroid gland<sup>[9]</sup>. Taken together it is hypothesized that the production of GADA in patients with APS3 might be attributable to a polyclonal activation of the autoimmune system in AITD and persistent positivity for GAD65A might be associated with increased risk to develop anti-thyroid autoimmunity.

It is unknown whether organ specific autoantibodies are directly involved in the pathogenesis of AITD or whether they are just secondary to tissue destruction by thyroid-infiltrating T-cells<sup>[10]</sup>. In our case, TGAb turned positive 12.5 years after the onset of type 1 diabetes. It has been questioned whether TGAb provide further diagnostic information as compared to the TPOAb in the diagnosis of AITD<sup>[3,11,12]</sup>. However, it has been reported that female gender, older (adolescence) onset, longer duration of diabetes, and positivity for GADA were risk factors for the development of thyroid disorders<sup>[3,5]</sup>. In addition, it is generally accepted that a certain type of HLA is involved in the development of type 1 diabetes and AITD<sup>[13,14]</sup>.

An association between AITD and HLA-DR3<sup>[15]</sup> and DRB1\*04-DQB1\*03:01<sup>[16]</sup> has been reported in Caucasian population. In addition, HLA-DRB1\*03:01-DQB1\*02:01 and DR4-DQB1\*03:02 has also been reported to contribute to both type 1 diabetes and AITD in a study of families with both diseases<sup>[17,18]</sup>. Therefore, our patient, who has HLA-DRB1\*03:01-DQB1\*02:01 and DRB1\*04:01-DQB1\*03:02 haplotypes, might be at high risk for the later development of AITD. Therefore, thyroid function test should be performed regularly in this patient for possible early diagnosis of thyroid disorders, although thyroid function is still normal.

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# World Journal of *Diabetes*

*World J Diabetes* 2013 December 15; 4(6): 231-377

Volume End



**EDITORIAL**

- 231 Diabetic macular edema: Current management 2013  
*Arevalo JF*

**FIELD OF VISION**

- 234 Betatrophin: A liver-derived hormone for the pancreatic  $\beta$ -cell proliferation  
*Raghow R*

**REVIEW**

- 238 Effect of obstructive sleep apnea on type 2 diabetes mellitus: A comprehensive literature review  
*Nannapaneni S, Ramar K, Surani S*
- 245 Diabetic nephropathy: Is it time yet for routine kidney biopsy?  
*Gonzalez Suarez ML, Thomas DB, Barisoni L, Fornoni A*
- 256 Prescribing physical activity to prevent and manage gestational diabetes  
*Colberg SR, Castorino K, Jovanovič L*

**TOPIC HIGHLIGHT**

- 263 Down-regulation of pancreatic transcription factors and incretin receptors in type 2 diabetes  
*Kaneto H, Matsuoka TA*
- 270 Type 2 diabetes mellitus in children and adolescents  
*Reinehr T*
- 282 Therapeutic effects of sericin on diabetic keratopathy in Otsuka Long-Evans Tokushima Fatty rats  
*Nagai N, Ito Y*
- 290 Classification of diabetic retinopathy and diabetic macular edema  
*Wu L, Fernandez-Loaiza P, Sauma J, Hernandez-Bogantes E, Masis M*
- 295 Clinical trials on corticosteroids for diabetic macular edema  
*Al Dhibi HA, Arevalo JF*
- 303 Vascular endothelial growth factor trap-eye (Aflibercept) for the management of diabetic macular edema  
*Moradi A, Sepah YJ, Sadiq MA, Nasir H, Kherani S, Sophie S, Do DV, Nguyen QD*

**310** Ranibizumab in diabetic macular edema

*Krispel C, Rodrigues M, Xin X, Sodhi A*

**319** Enzymatic vitrectomy for diabetic retinopathy and diabetic macular edema

*Diaz-Llopis M, Udaondo P, Millán JM, Arevalo JF*

**324** Diabetic macular edema: New promising therapies

*Shamsi HNA, Masaud JS, Ghazi NG*

**ORIGINAL ARTICLE**

**339** Changes of phasic and tonic smooth muscle function of jejunum in type 2 diabetic Goto-Kakizaki rats

*Zhao JB, Chen PM, Gregersen H*

**BRIEF ARTICLE**

**349** Prevalence of normoglycemic, prediabetic and diabetic A1c levels

*Aponte J*

**358** Vildagliptin vs sulfonylurea in indian muslim diabetes patients fasting during Ramadan

*Shete A, Shaikh A, Nayeem KJ, Rodrigues L, Ali MSS, Shah P, Khanna R, Majid S, Rasheed SA, Shaikh S, Rahman T*

**365** Effect of hypolipidemic treatment on glycemic profile in patients with mixed dyslipidemia

*Kei A, Liberopoulos E, Elisaf M*

**372** Influence of physiological and supraphysiological hyperinsulinemia on skin microcirculation in healthy volunteers

*Krčma M, Čechurová D, Brožová J, Jankovec Z, Lacigová S, Žourek M, Rušavý Z*

**APPENDIX** I-V Instructions to authors

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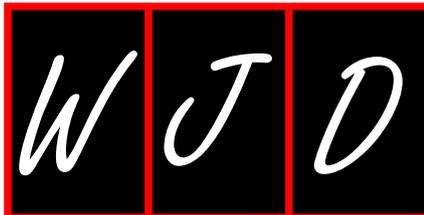
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## Diabetic macular edema: Current management 2013

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### Abstract

Diabetic retinopathy (DR) is the leading cause of vision loss of working-age adults, and diabetic macular edema (DME) is the most frequent cause of vision loss related to diabetes. The Wisconsin Epidemiologic Study of Diabetic Retinopathy found the 14-year incidence of DME in type 1 diabetics to be 26%. Similarly the Diabetes Control and Complications Trial reported that 27% of type 1 diabetic patients develop DME within 9 years of onset. The most common type of diabetes, type 2, is strongly associated with obesity and a sedentary lifestyle. An even higher incidence of macular edema has been reported in older patients with type 2 diabetes. Within the last 5 years, the use of intravitreal corticosteroids and intravitreal anti-vascular endothelial growth factor (VEGF) agents have come into clinical practice for the management of DME and several recent randomized clinical trials have shown improved effectiveness of ranibizumab compared to focal/grid laser. In this theme issue, we discuss the classification of DR and the treatment options currently available for the treatment of DME including corticosteroids, anti-VEGF agents, combined therapy, enzymatic vitrectomy (vitrealysis), and new therapies.

### DIABETIC MACULAR EDEMA

An estimated 347 million people are affected by diabetes worldwide in 2011, of those 138 million live in China and India and another 36 million in the United States and Russia. The number of people with diabetes is expected to double by 2030<sup>[1]</sup>. Diabetic retinopathy (DR) is the leading cause of vision loss of working-age adults<sup>[2]</sup>, and diabetic macular edema (DME) is the most frequent cause of vision loss related to diabetes. The Wisconsin Epidemiologic Study of Diabetic Retinopathy found the 14-year incidence of DME in type 1 diabetics to be 26%<sup>[3]</sup>. Similarly the Diabetes Control and Complications Trial (DCCT) reported that 27% of type 1 diabetic patients develop DME within 9 years of onset<sup>[4]</sup>. The most common type of diabetes, type 2, is strongly associated with obesity and a sedentary lifestyle. An even higher incidence of macular edema has been reported in older patients with type 2 diabetes<sup>[5]</sup>.

Argon laser photocoagulation has been the mainstay of treatment for macular edema since the publication of the results of the Early Treatment Diabetic Retinopathy Study (ETDRS), which showed an approximate 50% reduction in the rate of moderate vision loss at 3 years following laser photocoagulation compared to no treatment<sup>[6]</sup>. However, for patients with center involved macu-

lar edema, the risk of moderate vision loss at 3 years remained 15% with treatment<sup>[6]</sup>. Since the publication of ETDRS, the DCCT and United Kingdom Prospective Diabetes Study have demonstrated that tight glycemic and blood pressure control decrease the risk of microvascular complications of diabetes, including DR and vision loss<sup>[4,7-10]</sup>. As intensive blood pressure and blood sugar control have become the standard of care, visual outcomes have improved, but recent studies from the Diabetic Retinopathy Clinical Research Network indicate that even with the guidelines of tight glycemic and blood pressure control, 12%-13% of patients with foveal centered diabetic macular edema who undergo focal/grid laser lose 10 or more ETDRS letters after 2-3 years of follow-up. Additionally, with a baseline median vision of 20/50-20/63, only 36%-44% of patients gained 10 or more ETDRS letters at 2-3 years of follow-up, indicating the need for improved treatment modalities<sup>[11-13]</sup>. The outcomes may be even worse in developing countries where glycemic and blood pressure are poorly controlled.

Within the last 5 years, the use of intravitreal corticosteroids and intravitreal anti-vascular endothelial growth factor (VEGF) agents have come into clinical practice for the management of DME and several recent randomized clinical trials have shown improved effectiveness of ranibizumab compared to focal/grid laser<sup>[12-15]</sup>. In this theme issue, we discuss the classification of diabetic retinopathy and the treatment options currently available for the treatment of diabetic macular edema including corticosteroids, anti-vascular endothelial growth factor agents, combined therapy, enzymatic vitrectomy (vitrectomy), and new therapies.

Wu *et al*<sup>[16]</sup> give as the classification of diabetic retinopathy and DME. It is imperative that retina specialists, general ophthalmologists, internists, and endocrinologists learn to speak the same language in order to provide patients with the best care they deserve. Dhibi *et al*<sup>[17]</sup> review how corticosteroids have emerged as an alternative therapy for persistent DME or refractory to conventional laser photocoagulation and other modalities, due to anti-inflammatory, anti-VEGF and anti-proliferative effects. Many studies have demonstrated the beneficial therapeutic effect of corticosteroids with improvement to both retinal thickness and visual acuity (VA) in short-term on the treatment of DME. Peribulbar and intravitreal injections have been used to deliver steroids for DME with frequent injections due to the chronic and recurrent nature of the disease. Steroid-related side effects include elevated intraocular pressure, cataract, and injection related complications such as endophthalmitis, vitreous hemorrhage, and retinal detachment particularly with intravitreal steroid injections. In order to reduce the risks, complications, and frequent dosing of intravitreal steroids, intravitreal implants have been developed recently to provide a sustained release of corticosteroids and reduce repeated intravitreal injections for the management of DME. Stefanini *et al*<sup>[18]</sup> discuss the current status of the use of bevacizumab for the management of DME.

There is a strong evidence supporting that intravitreal bevacizumab injection has a good cost-effective profile in the management of DME and may be associated to laser photocoagulation; however, its clinical superiority regarding the duration of DME regression as well as the improvement of best-corrected VA compared to intravitreal ranibizumab and other intravitreal anti-VEGF therapies are still unclear and deserves further investigation. Moradi *et al*<sup>[19]</sup> review the treatment of DME using the first humanized monoclonal antibody targeting VEGF that has been FDA-approved for the use in the eye, ranibizumab. Krispel *et al*<sup>[20]</sup> discuss VEGF Trap-Eye (Aflibercept) for the Management of DME. Aflibercept presents a potential exciting new addition to the armamentarium of current VEGF antagonists available for the treatment of DME and other retinal vascular diseases. However, further studies are indicated to confirm the role, safety, and efficacy of aflibercept for DME. Diaz-Llopis *et al*<sup>[21]</sup> review their experience on enzymatic vitreolysis for diabetic retinopathy and DME. Enzymatic vitreolysis could be considered a good therapeutic alternative in diabetic retinopathy and macular edema. Finally, Al Shamsi *et al*<sup>[22]</sup> review new promising therapies for DME. The treatment of diabetic macular edema is rapidly evolving. The era of laser therapy is being quickly replaced by an era of pharmacotherapy. Several pharmacotherapies have been recently developed for the treatment of retinal vascular diseases such as diabetic macular edema. Several intravitreal injections or sustained delivery devices have undergone phase 3 testing while others are currently being evaluated. The results of clinical trials have shown the superiority of some of these agents to laser therapy. However, with the availability of several of these newer agents, it may be difficult to individualize treatment options especially those patients respond differently to various therapies. As such, more effort is still needed in order to determine the best treatment regimen for a given patient.

The etiology of DME is multifactorial. Therefore, the study of the important aspects of the pathogenesis and molecular pathways involved in the development of DME has led to the development of improved therapies for DME that have come into use in clinical practice. Additional promising therapeutic agents are currently being evaluated in clinical trials and additional molecular targets are being evaluated. Combined therapies targeting multiple pathways may yield synergistic treatment responses as several cytokines may be involved in the development of DME.

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## Betatrophin: A liver-derived hormone for the pancreatic $\beta$ -cell proliferation

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### Abstract

The pancreatic  $\beta$ -cell failure which invariably accompanies insulin resistance in the liver and skeletal muscle is a hallmark of type-2 diabetes mellitus (T2DM). The persistent hyperglycemia of T2DM is often treated with anti-diabetic drugs with or without subcutaneous insulin injections, neither of which mimic the physiological glycemic control seen in individuals with fully functional pancreas. A sought after goal for the treatment of T2DM has been to harness the regenerative potential of pancreatic  $\beta$ -cells that might obviate a need for exogenous insulin injections. A new study towards attaining this aim was reported by Yi *et al*, who have characterized a liver-derived protein, named betatrophin, capable of inducing pancreatic  $\beta$ -cell proliferation in mice. Using a variety of *in vitro* and *in vivo* methods, Yi *et al*, have shown that betatrophin was expressed mainly in the liver and adipose tissue of mice. Exogenous expression of betatrophin in the liver led to dramatic increase in the pancreatic  $\beta$ -cell mass and higher output of insulin in mice that also concomitantly elicited improved glucose tolerance. The authors discovered that betatrophin was also present in the human plasma. Surprisingly, betatrophin has been previously described by three other names, *i.e.*, re-feeding-induced fat and liver protein, lipasin and atypical angiotensin-like 8, by three inde-

pendent laboratories, as nutritionally regulated liver-enriched factors that control serum triglyceride levels and lipid metabolism. Yi *et al* demonstration of betatrophin, as a circulating hormone that regulates  $\beta$ -cell proliferation, if successfully translated in the clinic, holds the potential to change the course of current therapies for diabetes.

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**Key words:** Type 2 diabetes mellitus; Insulin resistance; Liver-derived betatrophin; Pancreas  $\beta$ -cell regeneration

**Core tip:** Yi *et al* have characterized a secreted protein named betatrophin that potently regulates  $\beta$ -cell proliferation. Evidently, betatrophin is identical to re-feeding-induced fat and liver protein, lipasin and atypical angiotensin-like 8, all of which were characterized as regulators of lipid homeostasis, by three independent groups of investigators. Yi *et al* were the first to demonstrate that hepatic expression of betatrophin in mice caused a dramatic surge in proliferation of the pancreatic  $\beta$ -cells with a concomitant improvement of their glucose tolerance. The discovery of a circulating hormone that specifically targets  $\beta$ -cell proliferation is a promising development towards a better clinical management of diabetes.

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### COMMENTARY ON HOT TOPICS

Chronic obesity associated type 2 diabetes mellitus (T2DM) is emerging as a global healthcare crisis (World Health Organization Global Infobase: data on over-

weight and obesity mean body mass index, healthy diets and physical inactivity; [www.who.int/mediacentre/](http://www.who.int/mediacentre/)). If the present trends continue nearly 350 million people worldwide will develop T2DM by the year 2030<sup>[1]</sup>. Insulin resistance in the skeletal muscle and liver accompanied by a “burnt-out” pancreas, as judged by its functional decline and reduced numbers of  $\beta$ -cells, is a hallmark of diabetes<sup>[2-4]</sup>. The underlying etiology of insulin resistance largely reflects a combination of chronic tissue inflammation and pancreatic  $\beta$ -cell failure<sup>[3,5]</sup>. Current therapies for the treatment of T2DM commonly include insulin sensitizers (*e.g.*, metformin and pioglitazone), with or without insulin secretagogues (*e.g.*, glyburide), or injectable insulin<sup>[6]</sup>. Decades of clinical experience have proven that current drug regimens to manage T2DM rarely achieve the physiological glycemic control elicited by a fully functional pancreas<sup>[6]</sup>. As a consequence, the cardiovascular, renal and neurological complications of poorly controlled blood sugar continue to be a major challenge in the management of T2DM<sup>[6]</sup>.

A long sought after but unrealized goal of treatment of T2DM is to replenish the loss of  $\beta$ -cell mass in the diabetic pancreas to restore normal glycemic control. Therefore, in recent years much effort has been focused on the physiological and pathological mechanisms that regulate the biogenesis, survival and apoptosis of the pancreatic  $\beta$ -cells. The pancreatic mass and  $\beta$ -cell numbers increase rapidly during embryogenesis and in the neonatal period, but the rate of replication of  $\beta$ -cells slows down dramatically in adult rodents and humans<sup>[7-9]</sup>. This is in contrast to a number of physiological (*e.g.*, gestation-induced diabetes) and pathological (*e.g.*, obesity-associated T2DM or pancreatic injury) states of insulin resistance during which pancreatic  $\beta$ -cells elicit enhanced rates of proliferation<sup>[8]</sup>. These observations have prompted a number of investigators to search for and identify a plethora of cell-intrinsic (*e.g.*, cyclin D1/D2; CDK-4, nuclear factors of activated T cells, Menin and p53) and systemic [glucagon-like peptide-1, glucose-dependent insulin-tropic polypeptide, leptin, adiponectin, interleukin (IL)-1, IL-6, tumor necrosis factor-1, fibroblast growth factor-21 and serotonin] factors that are presumed to control  $\beta$ -cell proliferation<sup>[8]</sup>. However, based on a rigorous functional assessment of the varied factors purported to control  $\beta$ -cell proliferation it is apparent that most of these factors lack sufficient potency and/or  $\beta$ -cell specificity for them to be warranted as serious candidates for drug development.

A number of recent observations in murine models of insulin resistance and obesity (*e.g.*, leptin-deficient *ob/ob* mice) appear to offer a way out of the current predicament. It has been reported that in a number of murine models of severe insulin resistance there is an apparent increase in pancreatic mass. The increased pancreatic mass seen under these conditions have reignited a search for systemic factor that might control proliferation of  $\beta$ -cells. The strongest evidence in favor of a putative factor(s) produced by the liver and released

in circulation originally came from Ronald Kahn's laboratory<sup>[10,11]</sup>. Using liver-specific insulin receptor knockout (LIRKO) mice, these authors reported the induction of a humeral factor involved in crosstalk between liver and pancreatic islets to trigger  $\beta$ -cells to multiply. Subsequently, El Ouaamari *et al*<sup>[12]</sup> applied the techniques of parabiosis, tissue transplantation, and *in vitro* islet culture in LIRKO mice to provide additional evidence for a non-neural and non-cell-autonomous factor(s) capable of inducing  $\beta$ -cell proliferation.

A parallel line of investigation in another model of obesity and insulin resistance also hinted at the existence of inter-organ metabolic communication between liver and pancreas. It had been suspected for some time that the livers of obese animals elicit enhanced activation of mitogen-activated protein kinase (MAPK) pathway. To investigate if MAPK signaling was involved in inter-organ relay of information between liver and pancreas, Imai *et al*<sup>[13]</sup> expressed a constitutively active extracellular regulated kinase (ca-ERK) in the livers of mice. Consistent with their hypothesis, ERK activation in the liver induced an obesity-associated metabolic signal(s) that was relayed *via* neuronal circuits to induce pancreatic  $\beta$ -cell proliferation. A number of additional studies have also hinted at the existence of a circulating factor(s) that might induce  $\beta$ -cell proliferation<sup>[10,14]</sup>. These tantalizing observations in experimental models of obesity notwithstanding, the identity of the putative liver-derived molecule(s) responsible for increased pancreatic  $\beta$ -cell mass and their implications for humans remain elusive. Apparently, Yi *et al*<sup>[15]</sup> were inspired by the tantalizing observations in LIRKO or ca-ERK mice to mount a systematic search for a putative liver-derived pancreatic  $\beta$ -cell growth factor produced in insulin resistance states.

Yi *et al*<sup>[15]</sup> began by co-opting a previously described model of insulin resistance in rat caused by infusion of S961, an insulin receptor (IR) antagonist peptide<sup>[16]</sup>. The authors delivered S961 by an osmotic pump and noted that S961 infusion caused dose-dependent peripheral insulin resistance and hyperglycemia that were accompanied by a dose-dependent surge in plasma insulin. These findings led the authors to posit that the observed surge in plasma insulin in mice most likely resulted from a compensatory increase in the numbers of pancreatic  $\beta$ -cells as a direct result of S961-induced insulin resistance. The authors experimentally tested this hypothesis and stained pancreatic sections of normal and S961-treated mice with specific antibodies to assess cell division [*e.g.*, Ki67 and Proliferating Cell Nuclear Antigen (PCNA)]; insulin- and nuclear Nkx6.1-specific antibodies were used to specifically stain the  $\beta$ -cells in the pancreatic islets. The histological assessments of pancreas from control and insulin-resistant mice were further complimented by quantitative polymerase chain reaction (qPCR) to measure expression of cell cycle specific genes (*e.g.*, cyclins, CDKs and E2Fs). Based on these analyses the authors concluded that S961 infusion-mediated insulin resistance in mice was accompanied with a potent (about 12-fold) and preferential

induction of cell proliferation.

Since a direct exposure of pancreatic islets to S961 did not cause  $\beta$ -cell proliferation, Yi *et al*<sup>[15]</sup> reasoned that the IR antagonist acted by altering gene expression in insulin sensitive tissues, other than pancreas, and potential inter-organ relay of signals indirectly impinged on the mechanism of pancreatic  $\beta$ -cell proliferation. To test this hypothesis, the authors analyzed global gene expression in untreated control and S961-treated liver, adipose tissue, skeletal muscle and pancreatic  $\beta$ -cells. The analyses of gene expression by microarrays revealed that a number of mRNAs were specifically induced in the liver and white adipose tissue (WAT) of S961-infused mice. Yi *et al*<sup>[15]</sup> chose a candidate gene from microarray analysis for more detailed *in silico* and experimental studies. They discovered that betatrophin mRNA was specifically induced by S961 in the liver and WAT of mice but not in skeletal muscle and pancreatic  $\beta$ -cells. The authors expressed exogenous betatrophin in the liver and detected the recombinant betatrophin protein in the serum. Interestingly, an *in silico* analysis of the putative betatrophin gene/transcript revealed that these had been annotated by other names in the mouse and human genomes. It became obvious from these analyses that *Gm6484* (gene) and EG624219 (protein) as annotated in the mouse genome and *C19orf80* (gene) and TD26 (hepatocellular carcinoma-associated protein), annotated in the human genome described the same genetic entity<sup>[17]</sup>.

Yi *et al*<sup>[15]</sup> noted that the expression of betatrophin mRNA was the most abundant in the liver and WAT of mice. Similarly, the expression of betatrophin was more than 250-fold higher in the human liver compared with WAT, heart, brain, pancreas or skeletal muscle. The authors extended their observations in S961-infused mice by exploring the expression of betatrophin in a number of other well-studied murine models of insulin resistance. Consistent with their hypothesis that betatrophin expression was a compensatory response to insulin resistance, Yi *et al*<sup>[15]</sup> observed that the livers of *db/db* and *ob/ob* mice expressed 3-4-fold higher levels of betatrophin mRNA. Similarly, a about 20-fold surge in hepatic expression of betatrophin mRNA was seen in mice during gestation, a time known to be associated with insulin resistance, accompanied with pancreatic  $\beta$ -cell expansion. Based on these observations the authors concluded that a state of insulin resistance was specifically involved in the induction of betatrophin gene expression. This conclusion was further bolstered by authors' observation that regeneration response of pancreas after its exposure to diphtheria toxin did not lead to induction of betatrophin.

As predicted by *in silico* analysis, Myc-tagged betatrophin expressed in Hepa1-6 or 293T cells behaved as a secreted protein, as judged by its intracellular location in vesicle-like structures, and its presence in the culture medium. Similarly, the hepatic expression of Myc-tagged betatrophin led to its appearance in circulation and was accompanied by a robust increase in the rate of replication of  $\beta$ -cells (17 to 33-fold). To examine the specificity

of betatrophin action on  $\beta$ -cells, Yi *et al*<sup>[15]</sup> stained pancreatic sections with Ki67- and PCNA-specific antibodies, and used qPCR to quantify expression of cyclins, *CDKs*, *E2F1* and *E2F2* genes. These studies revealed that exogenous hepatic expression of betatrophin was highly specific for  $\beta$ -cell proliferation since no other exocrine or endocrine cell types in the pancreas underwent multiplication. Thus, betatrophin appears to be a *bona fide* pancreatic  $\beta$ -cell specific hormone.

Yi *et al*<sup>[15]</sup> experimentally assessed the authenticity and normalcy of the newly formed  $\beta$ -cells by a number of criteria. They observed that pancreatic islets from control and betatrophin-expressing mice elicited similar glucose induced insulin secretion. The insulin tolerance tests in control and betatrophin-producing mice showed that they were similarly insulin sensitive. The normal insulin sensitivity of the betatrophin-expressing mice was in a sharp contrast to the situation seen in S961-infused mice that elicited robust insulin resistance. Finally, Yi *et al*<sup>[15]</sup> discovered that mice with forced expression of betatrophin elicited lower fasting glucose levels and had improved scores on glucose tolerance tests. These observations are consistent with the authors' conclusion that betatrophin, a hormone capable of inducing  $\beta$ -cell proliferation, is specifically produced by the liver under conditions of insulin resistance. Furthermore, the functional phenotype of the newly made  $\beta$ -cell in response to a forced expression of betatrophin appeared to be normal.

The discovery of a circulating hormone capable of inducing  $\beta$ -cell proliferation fulfills a cherished goal of many investigators engaged in T2DM research<sup>[14]</sup>. My commentary on the publication of Yi *et al*<sup>[15]</sup>, would not be complete without a few words about the re-discovery of betatrophin in its current guise and its molecular characterization by three other names by three independent laboratories. Thus, the discovery of so-called *RIFL* (re-feeding induced fat and liver) was reported as an adipocyte-enriched insulin target gene with a role in lipid metabolism<sup>[18]</sup>. A related strategy to analyze differential gene expression by RNA-seq expression experiments in the liver and WAT of fasted and high fat-fed mice, lead Zhang<sup>[19]</sup> to discover *lipasin* as a nutritionally regulated inhibitor of lipoprotein lipase that was involved in regulating serum triglyceride levels. Finally, Quagliarini and co-authors characterized angiopoietin-like 8 (ANGPTL8) protein, a member of angiopoietin-like family of proteins<sup>[20]</sup> that was shown to regulate postprandial fatty acid metabolism; apparently, ANGPTL8 could not carry out this function in the absence of its association with ANGPTL3, a paralog of ANGPTL8<sup>[21]</sup>. In retrospect, it is obvious that betatrophin, *RIFL*, *lipasin* and *ANGPTL8* are one and the same gene/protein. However, all three reports that preceded Yi *et al*<sup>[15]</sup> had described these genes as regulators of fatty acid metabolism and lipid homeostasis. In light of these observations the question of how mechanistically betatrophin exerts its pleiotropic actions in different tissues under physi-

ological and pathological conditions becomes even more urgent<sup>[17]</sup>. The current progress notwithstanding, a fuller understanding of the varied functions attributed to betatrophin/RIFL/Lipasin/ANGPTL8 must be clarified before full therapeutic implications of Yi *et al*<sup>[15]</sup> findings are realized.

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## Effect of obstructive sleep apnea on type 2 diabetes mellitus: A comprehensive literature review

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### Abstract

Obstructive sleep apnea (OSA) is frequently associated with obesity and metabolic syndrome. Also frequently associated with metabolic syndrome is type 2 diabetes mellitus (T2DM). Therefore, it is common to find OSA and T2DM together in individuals with metabolic syndrome. Additionally, both OSA and T2DM have a common pathophysiological link with development of insulin resistance. Individuals with severe insulin resistance are likely to have inadequate glycemic control. Long standing poorly controlled T2DM is associated with debilitating microvascular complications such as retinopathy, nephropathy, neuropathy and macrovascular complications such as coronary artery and cerebrovascular disease. There is extensively published literature exploring the cause-effect relationship between OSA and T2DM. In this article we provide an in-depth review of the complex pathophysiological mechanisms linking OSA to T2DM. Specifically, this review focusses on the effect of OSA on the microvascular complications of T2DM such as retinopathy, nephropathy and neuropathy. Additionally, we review the current literature on the effect of continuous positive airway pressure use

in individuals with T2DM and OSA.

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**Key words:** Sleep apnea and diabetes; Obstructive sleep apnea and diabetic complications; Obstructive sleep apnea; Diabetic complications; Sleep related breathing disorder; Diabetes

**Core tip:** This manuscript addresses the effect of obstructive sleep apnea (OSA) on type 2 diabetes (T2DM) and its associated vascular complications. Specifically, this article provides a comprehensive review of the association between OSA and the microvascular complications of T2DM. Finally, a summary of the effect of the use of continuous positive airway pressure treatment in individuals with OSA and T2DM is reviewed.

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### INTRODUCTION

Obstructive sleep apnea (OSA) is a sleep related breathing disorder characterized by collapse of the upper airway leading to cessation of airflow in the setting of continued respiratory effort<sup>[1]</sup>. The resultant hypoxia leads to frequent arousals causing sleep fragmentation and symptoms of excessive daytime sleepiness. Also, sleep fragmentation increases sympathetic activity, which can increase blood sugar levels by decreasing insulin sensitivity and glucose effectiveness<sup>[2]</sup>.

According to the American Diabetes Association (ADA), diabetes results from a defect in insulin secretion, insulin action or a combination of both<sup>[3]</sup>. The current

global prevalence of diabetes is 135 million and is expected to be 300 million by 2025<sup>[4]</sup>. Obesity, male gender and older age are well known risk factors for development of OSA and these risk factors are also associated with the increased likelihood of developing type 2 diabetes mellitus (T2DM)<sup>[5]</sup>. There is a growing body of evidence describing the association between OSA, insulin resistance and the subsequent development of T2DM<sup>[6-11]</sup>. Also, the importance of effective treatment of OSA in individuals with T2DM has been well studied<sup>[12,13]</sup>. In spite of this, OSA remains an underdiagnosed and under-treated condition in individuals with T2DM<sup>[11-13]</sup>. This article provides a concise review of current literature on the relationship between OSA and T2DM, the effect of OSA on the secondary complications of T2DM, and the effect of OSA treatment on outcomes related to T2DM.

## OBSTRUCTIVE SLEEP APNEA AND ITS RELATIONSHIP TO GLUCOSE METABOLISM

OSA is known to induce a severe state of insulin resistance (which is a risk factor for cardiovascular disease even in the absence of T2DM), resulting in marked compensatory hyperinsulinemia and thereby increasing the requirement for higher doses of exogenous insulin<sup>[5,14]</sup>. Two recent studies suggested that sleep disordered breathing is independently associated with glucose intolerance and insulin resistance<sup>[12,15]</sup>. In this large population based cohort, the investigators found that individuals with OSA were more likely to have lower levels of insulin sensitivity (34% *vs* 54%,  $P \leq 0.0001$ ) and higher levels of fasting insulin production compared to those without OSA. The pathophysiological basis of hyperglycemia in OSA appears to be twofold; hypoxia and sleep fragmentation (Figure 1). The mechanisms involved in the development of hypoxia induced hyperglycemia and insulin resistance have been extensively studied<sup>[8-13]</sup>. Sympathomimetic hormone (epinephrine, nor-epinephrine and cortisol) levels were noted to be elevated in healthy volunteers subjected to transient hypoxia<sup>[16-19]</sup>. These studies demonstrate that hypoxia causes a significant elevation in epinephrine levels leading to an increase in hepatic gluconeogenesis and decrease in skeletal muscle reuptake of glucose resulting in hyperglycemia. Additionally, the genesis of metabolic dysfunction in sleep disordered breathing likely involves several distinct but synergistic processes, including activation of the sympathetic nervous system, increase in oxidative stress, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, and low-grade systemic inflammation<sup>[9]</sup>. Elevation of systemic inflammatory markers such as tumor necrosis factor (TNF)- $\alpha$ , interleukin-6 (IL-6), high-sensitivity C-reactive protein (hsCRP), fibrinogen and uric acid, all of which could be secondary to the combined interactions of obesity, hyperglycemia and nocturnal hypoxia, are also possible contributing factors<sup>[20]</sup>. Elevation of these inflammatory markers leads to insulin resistance

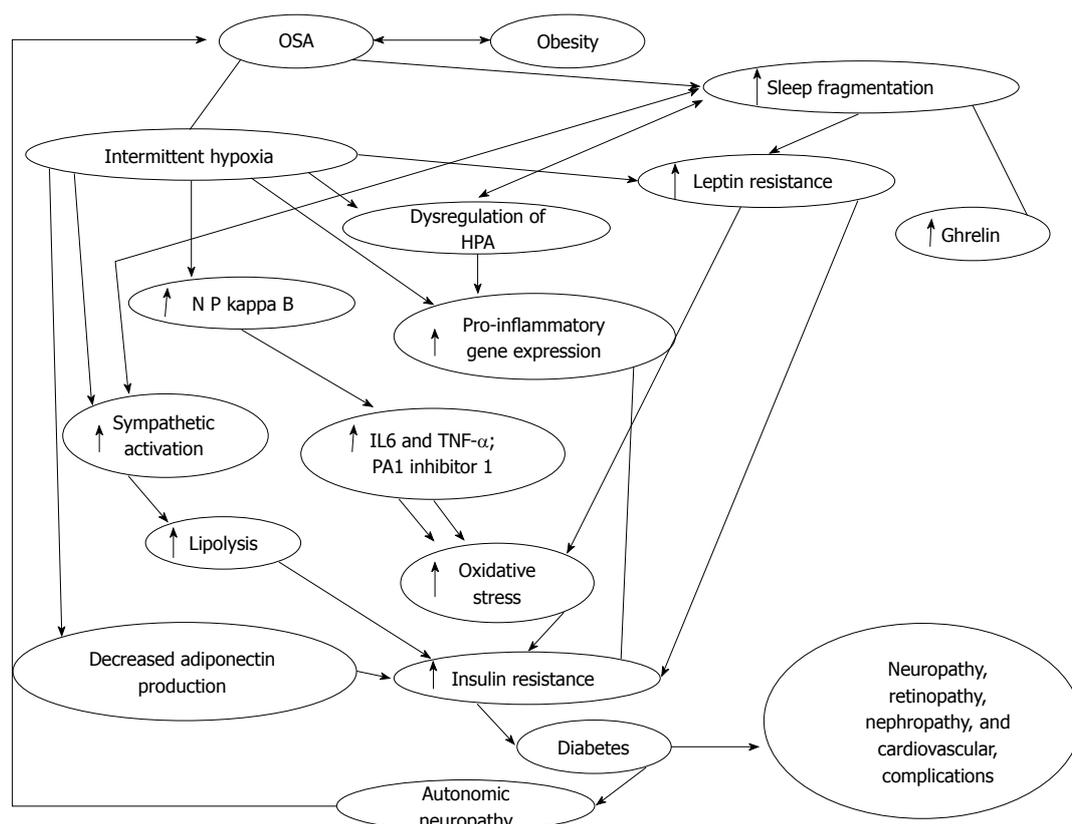
and impaired glucose utilization<sup>[21]</sup>. Furthermore, sleep fragmentation for as little as two nights has been shown to decrease insulin sensitivity and impair glucose metabolism<sup>[2]</sup>. Sleep fragmentation induced hyperglycemia and decreased insulin sensitivity appears to be mediated through alterations in sympathovagal balance, with a shift toward increased sympathetic nervous system activity during sleep and wakefulness<sup>[2]</sup>. These findings were also specifically studied in women with sleep apnea<sup>[15]</sup>. Women were noted to have a decrease in insulin sensitivity and beta-cell function, and higher loss of the Homeostasis Model Assessment (HOMA) product<sup>[15]</sup>. These two abnormalities were associated with poorer glycemic control and a 10% higher hemoglobin A1c (HbA1c) even after adjusting for intensive treatment for T2DM. In fact in this study, women with sleep apnea received more insulin than women without sleep apnea. Therefore, the relationship between OSA as a significant contributing factor to impaired glucose metabolism appears well proven.

## OBSTRUCTIVE SLEEP APNEA AND DIABETES MELLITUS TYPE 2

A vast body of literature has been published establishing the relationship between OSA and T2DM. Cross-sectional estimates from clinic populations and population studies suggest that up to 40% of patients with OSA have diabetes<sup>[7,22,23]</sup>, but the incidence of new diabetes in patients with OSA is not known. Likewise, in patients who are known to have diabetes, the prevalence of OSA may be up to 23%<sup>[11]</sup>, and the prevalence of some form of sleep disordered breathing (SDB) may be as high as 58%<sup>[24]</sup>. In patients with established T2DM there is a significant relationship between SDB and fasting insulin, glucose, and HbA1c levels, that is independent of obesity as determined by the waist-hip ratio<sup>[16]</sup>. Although there is now convincing evidence of the association between OSA and decreased insulin sensitivity, the exact pathophysiological mechanism linking OSA as a causative factor of T2DM remains elusive. The converse (T2DM causing OSA) is postulated to involve the dysregulation of the autonomic nervous system leading to SDB. Diabetic autonomic neuropathy likely leads to ventilatory dysfunction through decreased heart rate variability and impaired central control of breathing leading to SDB<sup>[25-29]</sup>. One study showed that 25% of diabetic individuals with autonomic neuropathy have sleep apnea, a proportion greater than in diabetic subjects without autonomic neuropathy<sup>[25]</sup>. Based on the current body of literature it is therefore possible to postulate that there is a strong relationship between T2DM and OSA.

## RELATIONSHIP BETWEEN OBSTRUCTIVE SLEEP APNEA AND MICROVASCULAR COMPLICATIONS OF DIABETES MELLITUS

Long standing poorly controlled T2DM is associated



**Figure 1** Flow diagram demonstrating interplay between obstructive sleep apnea, intermittent hypoxia, sleep fragmentation and diabetes. OSA: Obstructive sleep apnea; NF-κB: Nuclear factor kappa B; PA1: Plasminogen activator inhibitor 1; HPA: Hypothalamic-pituitary axis; IL: Interleukin; TNF-α: Tumor necrosis factor-α. Modified from Ref [58].

with the development of microvascular complications such as retinopathy, nephropathy, neuropathy and macrovascular disease such as coronary artery and cerebrovascular disease. In this section, we review the literature on the association between OSA and the risk of developing T2DM its related microvascular complications. The macrovascular complications of T2DM have been well recognized. OSA leads to increase in insulin resistance and T2DM, which in turn can lead to increase in the inflammatory markers leading to cardiovascular complications. Studies have shown that treatment with continuous positive airway pressure (CPAP) can decrease the levels of IL-6 and CRP, which in turn can lead to decrease in inflammation and reduce vascular complications<sup>[6,30]</sup>. Several studies have demonstrated an association between OSA and cardiovascular abnormalities<sup>[15,31]</sup>, and improvement with CPAP therapy. Discussing this is beyond the scope of this review. Here we discuss briefly the role of OSA in diabetic retinopathy, neuropathy, nephropathy and insulin resistance and its treatment. The complex interplay of sleep and diabetes demonstrating interplay between OSA, T2DM, intermittent hypoxia, sleep fragmentation and insulin resistance is shown in Figure 1.

## DIABETIC RETINOPATHY

The relationship between diabetes mellitus and the

development of proliferative retinopathy is well established. Although one of the first studies<sup>[32]</sup> to assess the potential relationship between OSA and diabetic retinopathy did not find a strong association, other recent studies have found<sup>[33-36]</sup> that OSA remained an independent predictor of proliferative retinopathy even after adjusting for conventional risk factors and novel biomarkers for diabetic retinopathy. Also, in individuals with OSA, where hypertension and obesity are comorbid to T2DM, there appears to be an increased risk of proliferative retinopathy<sup>[34]</sup>. Diabetic retinopathy is mediated by high levels of serum vascular endothelial growth factors and other biomarkers<sup>[33,37,38]</sup>. Similarly, the increased risk of diabetic retinopathy in OSA appears to be mediated by elevated levels of inflammatory markers, reduced endothelial regulatory function and increased insulin resistance<sup>[39,40]</sup>. A recent study by Fujita *et al*<sup>[41]</sup> noted an increase in the levels of inflammatory markers such as acylation stimulating protein, high sensitive CRP and components of the membrane attack complex (which leads to activation of alternative complement pathway) in obese T2DM patients with retinopathy. As a result, complement mediated activation of inflammation likely leads to acceleration of diabetic microangiopathy leading to development and worsening of diabetic retinopathy. Previous studies<sup>[42,43]</sup> looking in to complement activation in individuals with OSA have noted increased levels of C3 and a decrease in the levels of IgM and NK cell

percentage. All this data strongly points towards a strong relationship between OSA and increased risk of proliferative retinopathy in individuals with OSA and T2DM.

## DIABETIC NEPHROPATHY

Diabetes Mellitus is the leading cause of end stage renal disease<sup>[44]</sup>. Nephropathy in T2DM is postulated to be mediated through an increase in activity of angiotensin II, platelet derived growth factor, and thromboxane. These agents upregulate protein kinase c activity and this leads to activation of transforming growth factor-beta (TGF- $\beta$ ). TGF- $\beta$  leads to proliferation of extracellular matrix and glomerulosclerosis leading to diabetic nephropathy<sup>[45]</sup>. Progression of renal failure in the presence of OSA is likely secondary to hypoxia mediated increase in sympathetic activation and inflammatory cytokines<sup>[46]</sup>. Additionally, a recent study has also shown increased cystatin C levels in individuals with severe OSA<sup>[47]</sup>. Cystatin C has been recognized as an early biomarker for renal failure development and cardiovascular events<sup>[47]</sup>. Other studies have shown that the severity of OSA appears to be directly correlated to the degree of loss of renal function<sup>[48]</sup>. The literature on OSA leading to the progression of diabetic nephropathy is limited. A recent small study evaluated OSA as an independent risk factor for microalbuminuria and did not find any significant relationship<sup>[49]</sup>. This study consisted of a small sample of patients with moderate to severe OSA (Apnea hypopnea index > 15), and was underpowered to detect a difference. However, given the overall similarity in the pathophysiological mechanisms, it is likely that OSA may contribute to the development of diabetic nephropathy. Future larger population based studies will be necessary to better understand this relationship.

## PERIPHERAL NEUROPATHY

Peripheral neuropathy secondary to T2DM is very common, affecting 60%-70% of individuals with diabetes<sup>[44]</sup>. Severe forms of diabetic neuropathy are a major contributing cause of lower-extremity amputations<sup>[44]</sup>. The pathophysiologic mechanism of diabetic peripheral neuropathy appears to be complex; involving metabolic and ischemic pathways<sup>[50]</sup>. Metabolic factors mediated by hyperglycemia lead to abnormal nerve energy transport, impaired axonal transport, increased activity of the sorbitol pathway, non-enzymatic nerve protein glycosylation and abnormal myo-inositol metabolism. The ischemic pathway is mediated through thickening and hyalinization of the microvasculature wall leading to neuronal ischemia<sup>[50]</sup>. Previous studies have noted the relationship between OSA and peripheral neuropathy and the improvement in peripheral neuropathy with treatment of OSA using CPAP<sup>[51-54]</sup>. A recent study specifically looking into the relationship between OSA and diabetic peripheral neuropathy found that there was a fourfold increase in the odds of peripheral neuropathy in diabetic patients with OSA compared those without<sup>[36]</sup>. Also, noted was a

significant trend in the prevalence of diabetic peripheral neuropathy with lower levels of oxygen saturations. In the same study, individuals with OSA and diabetic peripheral neuropathy were found to have higher levels of nitrotyrosine and lipid peroxide levels compared to those without OSA. Nitrotyrosine and lipid peroxide leads to nitrosative and oxidative stress that reduces nerve perfusion, resulting in impairment of vascular reactivity of the epineurial arterioles<sup>[55]</sup>. Overall, there is stronger data now supporting the hypothesis that OSA is an independent risk factor for the development of diabetic peripheral neuropathy. A recent study-linking OSA to diabetic peripheral neuropathy has been postulated suggesting protein kinase, advanced glycation end products, hexosamine and polyol pathway each of those leading to microvascular complications including diabetic peripheral neuropathy<sup>[36]</sup>.

## TREATMENT OF OBSTRUCTIVE SLEEP APNEA AND OUTCOMES WITH IMPAIRED GLUCOSE TOLERANCE AND DIABETES MELLITUS

The strong association between insulin resistance and OSA would imply that treatment with CPAP should lead to improved glucose control in patients with T2DM. The effect of CPAP on glucose control has been variable<sup>[56]</sup>. Review of the literature found that most studies exploring the effect of CPAP on insulin sensitivity show a positive effect<sup>[57-65]</sup>, although other studies have not shown a significant improvement in hyperglycemia or level of insulin resistance<sup>[66-69]</sup>. The variability in results is likely due to methodological weaknesses such as small sample sizes, lack of adjustment for confounders and absence of polysomnographic evidence to support sleep duration. Other studies have shown that racial or ethnic differences among the study populations could contribute to variability in response to CPAP<sup>[70,71]</sup>. In one study the response to CPAP was significantly greater if the body mass index (BMI) was less than 30<sup>[60]</sup>. The likely explanation appears to be that obesity is a more important determinant of insulin resistance than OSA. The less obese the patients are, the greater is the improvement in insulin sensitivity brought about by CPAP treatment<sup>[60]</sup>. Another study observed a significant decrease in glycated hemoglobin values with CPAP, although there was no significant difference in fasting blood sugars and insulin resistance on a day-to-day basis<sup>[68,72]</sup>. The likely explanation for this finding is that glycated hemoglobin is a better marker of long term glucose control unlike blood glucose and insulin resistance, which can fluctuate on a day-to-day basis<sup>[68]</sup>. This suggests that monitoring HbA1c levels as a marker of efficacy of CPAP therapy is likely a better indicator than monitoring indices of insulin resistance or glucose utilization (*i.e.*, HOMA product). A recent randomized control study<sup>[64]</sup> noted a dose-response effect for both the severity of disease and

adherence to CPAP treatment. Insulin sensitivity was significantly better after treatment with CPAP in patients with severe OSA (Apnea hypopnea index  $\geq 30$ ) compared to patients with less severe OSA. Also, the same study revealed that each additional hour of active CPAP usage was associated with a significant improvement in insulin sensitivity. The reason for a lack of effect of CPAP on the less severe OSA patients is unknown. In spite of the variability in results of the studies, treatment with CPAP remains an important modality for patients with OSA to improve glucose metabolism. Treatment appears particularly important for patients with severe OSA and subjective symptoms of sleepiness. In light of the current results, clinicians should ensure patients with OSA are effectively treated, particularly with the epidemic of obesity and diabetes.

## CONCLUSION

There appears to be an association between OSA and the development of insulin resistance leading to impaired fasting glucose tests and the development of T2DM. Furthermore, data supports the development and progression of complications from long standing T2DM in the setting of inadequately treated OSA. There is an abundance of evidence demonstrating the link between OSA and impaired glucose metabolism. Irrespective of the direction of causality, the association between OSA and T2DM remains irrefutable. Therefore, in spite of the controversial nature of the data regarding the improvement in glycemic control with CPAP therapy, judgment should be reserved until long term rigorously conducted prospective studies can expand knowledge in this area. In the interim, although a strong recommendation for treatment of OSA with CPAP to control diabetes remains controversial, physicians should individualize their decisions based on the particular needs of their patients.

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## Diabetic nephropathy: Is it time yet for routine kidney biopsy?

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### Abstract

Diabetic nephropathy (DN) is one of the most important long-term complications of diabetes. Patients with diabetes and chronic kidney disease have an increased risk of all-cause mortality, cardiovascular mortality, and kidney failure. The clinical diagnosis of DN depends on the detection of microalbuminuria. This usually occurs after the first five years from the onset of diabetes, and predictors of DN development and progression are being studied but are not yet implemented into clinical practice. Diagnostic tests are useful tools to recognize onset, progression and response to therapeutic interventions. Microalbuminuria is an indicator of DN, and it is considered the only noninvasive marker of early onset. However, up to now there is no diagnostic tool that can predict which patients will develop DN before any damage is present. Pathological renal injury is hard to predict only with clinical and laboratory

findings. An accurate estimate of damage in DN can only be achieved by the histological analysis of tissue samples. At the present time, renal biopsy is indicated on patients with diabetes under the suspicion of the presence of nephropathies other than DN. Results from renal biopsies in patients with diabetes had made possible the classification of renal biopsies in three major groups associated with different prognostic features: diabetic nephropathy, non-diabetic renal disease (NDRD), and a superimposed non-diabetic condition on underlying diabetic nephropathy. In patients with type 2 diabetes with a higher degree of suspicion for NDRD, it is granted the need of a renal biopsy. It is important to identify and differentiate these pathologies at an early stage in order to prevent progression and potential complications. Therefore, a more extensive use of biopsy is advisable.

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**Key words:** Diabetic nephropathy; Kidney biopsy; Non-diabetic renal disease

**Core tip:** Diagnostic tests are useful to predict onset, progression and response to therapeutic interventions in diabetic nephropathy (DN). Renal biopsies help to classify renal diseases in three major groups associated with different prognostic features: diabetic nephropathy, non-diabetic nephropathy (NDRD), and a superimposed non-diabetic condition on underlying DN. Pathological renal damage is hard to predict only with clinical and laboratory findings. In patients with a higher degree of suspicion for NDRD, it is granted the need of a renal biopsy. For this reason, more studies are required to assess the routine use of kidney biopsies as a gold standard for diagnosis of diabetic nephropathy.

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## INTRODUCTION

Chronic kidney disease (CKD) is a worldwide public health problem, and one of the major causes of mortality in the United States. It is characterized by kidney damage for more than 3 mo; defined by structural or functional abnormalities of the kidney, with or without decreased estimated glomerular filtration rate (GFR); or GFR < 60 mL/min per 1.73 m<sup>2</sup> for more than 3 mo, with or without kidney damage<sup>[1]</sup>. Decreased GFR and albuminuria are indicators of major health outcomes of this condition, including end-stage renal disease (ESRD) and death<sup>[2]</sup>.

There is no definitive cure for this condition; and many of the patients may develop complications even before they are able to receive renal replacement therapies, including long term dialysis and kidney transplants. In 2010, more than 117000 patients started therapy for ESRD; the prevalent population reached 594000, from those a 74% (439560) required dialysis and 3% (17778) received kidney transplants<sup>[3]</sup>.

The most common cause of ESRD requiring dialysis is diabetes mellitus (DM). Up to 44% of patients with newly diagnosed ESRD cases also carry a diagnosis of diabetes<sup>[3]</sup>. According to the World Health Organization (WHO) in 2012, 347 million people suffered from DM; this is about 5% of the total population. Individuals with diabetes and CKD have an increased risk of all-cause mortality, cardiovascular mortality, and kidney failure<sup>[3-5]</sup>. WHO estimates that deaths related to diabetes will double from 2005 to 2030<sup>[4]</sup>.

## DEFINITION OF DIABETIC NEPHROPATHY AND WORLDWIDE IMPACT

Diabetic nephropathy (DN) is one of the most important long-term complications of diabetes. It is characterized by the development of proteinuria with a subsequent decline in glomerular filtration rate, which progresses over a long period of time, often for 10-20 years<sup>[3,6,7]</sup>.

Over the past 20 years, the prevalence of DN in the United States has increased in direct proportion to the prevalence of diabetes<sup>[6]</sup>. Although DN cases vary largely among countries; in average it develops in 30% to 40% of patients with diabetes<sup>[7]</sup>. The clinical diagnosis of DN usually depends on the detection of microalbuminuria (albumin excretion of more than 30 mg/g of creatinine in 2 out of 3 random urine samples collected in within a six month period)<sup>[8]</sup>. A subset of patients with microalbuminuria will develop advanced DN; referred as overt nephropathy, clinical nephropathy, proteinuria, or macroalbuminuria<sup>[9]</sup>. However, progression to microal-

**Table 1 Diabetic nephropathy stages**

Stage 1 Early hyperfunction and hypertrophy	ACR < 30 mg/g creatinine
Stage 2 Morphologic lesions without signs of clinical disease	ACR > 30 and < 300 mg/g creatinine
Stage 3 Microalbuminuria	ACR > 300 mg/g creatinine and/or persistent proteinuria with serum concentration of creatinine 2.0 mg/dL
Stage 4 Overt nephropathy	Serum concentration of creatinine 2.0 mg/dL with proteinuria
Stage 5 End-stage renal disease with uremia	On dialysis

ACR: Albumin to creatinine ratio.

buminuria usually occurs after five years from the onset of diabetes. Pathogenesis of the disease is multifactorial, *e.g.*, smoking, hyperglycemia, hypertension, male, genetic predisposition, advance age, retinopathy, macrovascular disease were the risk factors of diabetic nephropathy; and it involves genetic and environmental factors that affect multiple metabolic pathways not necessarily activated by hyperglycemia<sup>[10]</sup>.

## STAGES OF DIABETIC NEPHROPATHY

Progression of the diabetic nephropathy is divided in clinical stages depending on the duration of the disease<sup>[11-13]</sup> (Table 1). The first stage starts prior to any renal damage. It is characterized by renal vasodilation and hyperfiltration that occur early in the onset of diabetes. Several factors may lead to this hyperfiltration: including hyperglycemia, prostaglandins secretion and increased sodium/glucose reabsorption in the proximal tubule<sup>[14]</sup>. It has also been associated to increased urinary albumin excretion related to physical activity<sup>[15]</sup>.

During the second stage, morphologic lesions develop without signs of clinical disease. The earliest structural abnormality in diabetes is glomerular basement membrane (GBM) thickening. The kidney with early diabetes suffers significant hypertrophy; characterized by enlargement of the organ with a combination of hyperplasia and hypertrophy<sup>[16]</sup>. This occurs in nearly all patients 1.5 to 2.5 years after the onset of type 1 DM (T1DM). Nonspecific vascular or interstitial changes are prevalent in these patients. Mesangial expansion and occlusion of glomerular capillaries lead to a loss of available surface area for filtration and to a decline in function<sup>[14]</sup>.

Third stage is characterized by small amounts of albumin in the urine, not usually detected by conventional methods. This stage is also named incipient nephropathy<sup>[9]</sup>. A slow and gradual increase of albuminuria over the years is a prominent feature in this stage. According to the DCCT/EDIC Study, persistent microalbuminuria develops most frequently during the second decade after diagnosis of diabetes<sup>[6]</sup>. It reflects the existence of endothelial damage in the absence of specific renal lesions; and it is also associated with the beginning of advanced

renal pathology<sup>[7]</sup>. Microalbuminuria could also represent podocytes loss; as podocyte number in patients with type 2 DM (T2DM) correlates with the change of albuminuria over time<sup>[17]</sup>.

Although microalbuminuria has been considered a risk factor for macroalbuminuria, not all patients progress to this stage; some of them stay or even may regress to normoalbuminuria<sup>[18]</sup>. Microalbuminuria is considered to be predictive of progression to nephropathy in T2DM. However, that may not be the case in T1DM<sup>[14]</sup>. Normoalbuminuric patients with diabetes are extremely heterogeneous in renal function and structure<sup>[19]</sup>. Both, microalbuminuric and normoalbuminuric patients benefit from optimal glycemic control<sup>[20]</sup>; since it has been shown that about one third of the normoalbuminuric subjects develop diabetic nephropathy within few years after onset of diabetes<sup>[19,21-23]</sup>. The cause of albuminuria in patients without diabetic glomerulopathy is unclear. It might be related to early and very mild ultrastructural changes<sup>[24]</sup>.

Overt nephropathy is characterized by persistent albuminuria (UAE > 300 mg/d or > 500 mg/d urinary protein excretion) that usually accompanies a decrease in GFR<sup>[25]</sup>. Macroalbuminuria has been associated to the presence of proliferative retinopathy, coronary heart disease, and foot ulcers<sup>[14]</sup>. The prevalence of hypertension increases with higher levels of albuminuria<sup>[15]</sup>. Other risk factors to develop overt nephropathy include uncontrolled diabetes, smoking, advanced age and high lipids levels<sup>[26,27]</sup>. ESRD is defined by the presence of signs and symptoms of kidney failure requiring replacement therapy, regardless of the GFR level<sup>[1]</sup>. It has been described as an important independent predictor of hospitalization and death in adults with heart failure. The deterioration rate from one stage to the next one is 2% to 3% per year<sup>[28]</sup>.

## DIAGNOSTIC TOOLS FOR DIABETIC NEPHROPATHY

One of the main goals concerning the timely diagnosis of DN is to delay and if possible interrupt the natural course of this disease; from the progression of renal damage to ESRD in patients with diabetes. Diagnostic tests are useful tools to determine onset, progression and to predict response to therapeutic interventions.

### Current screening recommendations

Although not all patients with early renal involvement, such as microalbuminuria progress to macroalbuminuria and ESRD; the risk is higher among these patients. Some of them stay or even may regress to normoalbuminuria<sup>[18]</sup>. Ideally, it would be very useful to be able to predict which patients are at higher risk to develop ESRD, even before onset of DN. Unfortunately, at the present time, there is a lack of precise diagnostic tools that could definitively identify such patients<sup>[29]</sup>.

The National Kidney Foundation (NKF) and the American Diabetes Association (ADA) recommend that

patients with CKD and diabetes should be screened every year for DN. Screening should start 5 years after diagnosis in T1DM patients, and at the time of diagnosis in T2DM patients. This is done by measuring the urinary albumin/creatinine ratio in spot urine, serum creatinine and GFR<sup>[1]</sup>. Although UAE is routinely used to diagnose DN; in some cases, patients with diabetes have a decrease GFR with normal UAE. Both GFR and UAE, correlate with the severity of glomerular lesions, duration of diabetes, glycaemic control and genetic factors<sup>[30,31]</sup>.

Currently, microalbuminuria is considered the earlier noninvasive marker<sup>[32-34]</sup>. Patients with both elevated albuminuria and reduced GFR are at higher risk for a cardiovascular event<sup>[35]</sup>. This emphasizes the importance to detect microalbuminuria and a close follow up of especially in young patients with diabetes.

### Renal biopsy

Once the presence of albumin in the urine is confirmed, patients should undergo complete evaluation; including work-up for other etiologies. Renal diseases other than DN have been reported in patients with diabetes. DN usually develops 10 years after onset of T1DM<sup>[18]</sup>; however, in T2DM this is variable<sup>[23]</sup>.

An accurate estimate of damage in DN can only be achieved by the histological analysis of tissue samples<sup>[7]</sup>. Therefore, the kidney biopsy in patients with diabetes could represent a valuable procedure to establish the stage of the renal disease<sup>[36]</sup>. The relevance of this diagnostic tool is supported by the observation that when a renal biopsy is performed in patients with DM, results may vary from primary and secondary renal disease with changes unrelated to diabetes to changes of underlying DM<sup>[23]</sup>.

Some of the earliest lesions are characterized by the thickening of the GMB visualized under electron microscopy, but with no findings under light microscopy. The morphologic lesions in T1DM predominantly affect the glomeruli, with thickening of the GBM and mesangial expansion; although the podocytes, renal tubules, interstitium, and arterioles also undergo substantial changes, especially at later stages of disease<sup>[37,38]</sup>.

Nephropathy in patients with T2DM is associated with two distinctive patterns of glomerular pathology (nodular and non-nodular)<sup>[39]</sup>. Nodular type glomerulosclerosis (Kimmelstiel-Wilson nodules) was reported in 1936 by light microscopy. This lesion was initially identified as the only specific feature of DN<sup>[40]</sup>. It consists of nodular lesions containing areas of marked mesangial expansion forming large round fibrillar mesangial zones with palisading of mesangial nuclei around the periphery of the nodule and compression of the associated glomerular capillaries. Later on, diffuse type glomerulosclerosis was described as a different type of diabetic glomerular lesion<sup>[41]</sup>. All these diabetic glomerular changes are related to advanced or late DN associated to heavy proteinuria and/or decreased renal function. Arteriosclerosis is also frequently associated to diabetic glomerulopathy<sup>[42]</sup>.

It has been shown that there is not substantial difference in the injury caused in patients with T1DM in comparison to T2DM; and damages are considered basically similar in both types<sup>[29]</sup>. For this reason, there is a consensus classification combining type 1 and type 2 DN. It is divided into four classes of glomerular lesions. Class I: GMB thickening, composed of isolated GMB thickening and only mild, nonspecific changes by light microscopy that do not meet the criteria of classes II through IV. Class II: mesangial expansion; mild (IIa) or severe (IIb), without nodular sclerosis or global glomerulosclerosis in more than 50% of glomeruli. Class III: nodular sclerosis (Kimmelstiel-Wilson lesions); at least one glomerulus with nodular increase in mesangial matrix (Kimmelstiel-Wilson) without changes described in class IV. Class IV: advanced diabetic glomerulosclerosis, more than 50% global glomerulosclerosis with other clinical or pathologic evidence that sclerosis is caused by diabetic nephropathy<sup>[42,43]</sup> (Figure 1).

Podocyte injury is also an important feature of DN<sup>[16,44-49]</sup>, and podocyte loss (podocytopenia) is considered an independent predictor of DN progression in patients with T2DM<sup>[17]</sup>.

### Indications for kidney biopsy

There are no standardized criteria for kidney biopsy in patients with DM; therefore, currently the decision to perform one is made by the primary physician<sup>[50,51]</sup>. Nowadays, up to 25% of all renal biopsies are done in patients with DM<sup>[51]</sup>.

Rapid onset of proteinuria (regardless of the progression from microalbuminuria to macroalbuminuria), absence of retinopathy, presence of hematuria, active urinary sediment, rapid decrease of renal function, and suspicion of other nephropathies secondary to systemic disease, are some of the indications for renal biopsy<sup>[50,52]</sup>, *e.g.*, Nephrotic syndrome, Urinary abnormalities, Isolated hematuria, Nephritic syndrome, Rapid onset of renal insufficiency, Unexplained renal failure at presentation, and No retinopathy are major indications for kidney biopsy. Some authors had considered retinopathy as a highly specific indicator for DN<sup>[53,54]</sup>. On the other hand, other studies have shown to be a poor predictor of DN in T2DM<sup>[55,56]</sup>. For this reason, kidney biopsy may have an opportunity to be proven as a gold standard for diagnosis of DN<sup>[57]</sup>.

### Biopsy findings in patients with diabetes

Results from renal biopsies in patients with diabetes have made possible the classification of renal biopsies in three major groups associated with different prognostic features: DN, non-diabetic renal disease (NDRD), and a superimposed non-diabetic condition on underlying DN. There has been described a more rapid deterioration of renal function on patients with DN than with NDRD<sup>[53]</sup>. Patients with non-diabetic nephropathies, including extracapillary glomerulonephritis, minimal change glomerulopathy, cryoglobulinemic nephritis, non-diabetic

membranous glomerulopathy, focal glomerulosclerosis, and IgA nephropathy, among others, might be modified by therapy; and for this reason, it results important the detection of such histological patterns following an appropriate therapeutic management; as this could promote a better outcome in those patients<sup>[23]</sup>. The most common non-diabetic glomerulopathy found across reports in literature is IgA nephropathy<sup>[60-72]</sup>.

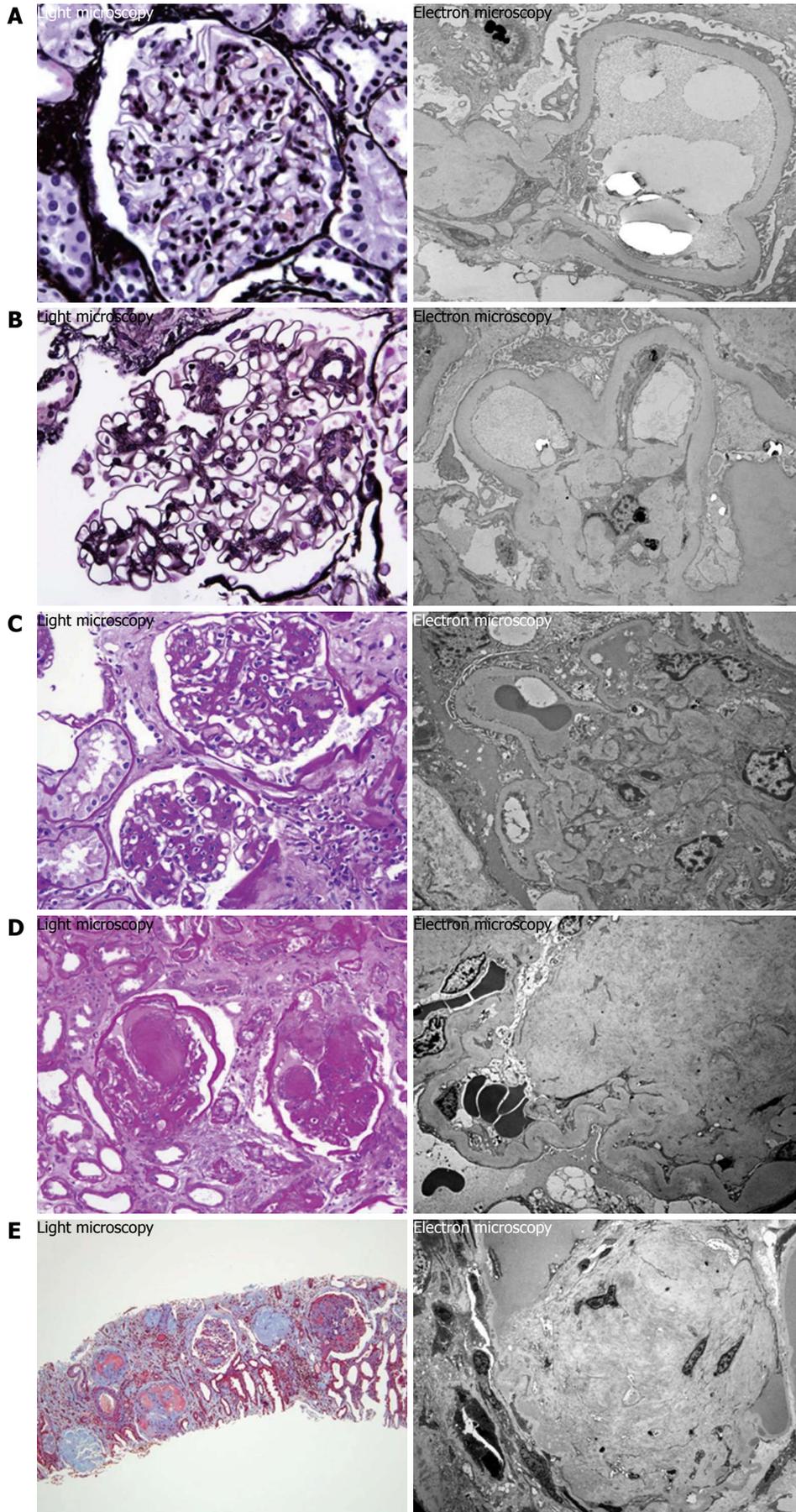
Clinical presentation varies among age groups. It is known that chronic nephritic syndrome is usually more common in young patients, and nephrotic syndrome and CKD in the elderly<sup>[50]</sup>. The average time of onset of nephropathy in patients with diabetes is about 7-10 years<sup>[18]</sup>.

It is important to emphasize that among patients with diabetes; especially in those with T2DM, renal complications may be frequently due to heterogeneous non-diabetic lesions. Reported NDRD varies in the literature from a range of 14%-82.9% (Table 2), regardless of geographic region or ethnicity. The wide range in these highly variable results could be explained by the heterogeneity of the populations in the studies done around the world. Nzerue *et al*<sup>[61]</sup>, reported a prevalence of NDRD among African-Americans with T2DM based on renal biopsy. They found DN alone in 42%, while NDRD was seen in 19%; the rest of the patients had a superimposed NDRD with DN<sup>[62]</sup>. Similar results were reported in two different studies in Japan<sup>[68,73]</sup>. On the other hand; Wong *et al*<sup>[53]</sup>, reported different results in the Chinese population which showed a predominance of NDRD. As the population sample used in some of these studies was larger (*i.e.*, studies with population sample of more than 70 patients), the proportion of DN *vs* NDRD become more homogeneous; resulting in 30% prevalence for each of these groups. Although there is a male predominance for DN in general, this is not statistically significant among the studies<sup>[58,62,71,74]</sup>.

Pathological renal damage is hard to predict only with clinical and laboratory findings<sup>[23,52,54]</sup>. In patients with T2DM with a higher degree of suspicion for NDRD, it is granted the need of a renal biopsy<sup>[53]</sup>. Therefore, a more extensive use of biopsy is advisable. There are other cases where is not routinely performed; for example in patients with T2DM and ESRD, especially in those who present with criteria for clinical diagnosis<sup>[57]</sup>.

### Complications of kidney biopsy

Kidneys are highly vascular organs; therefore the most common complications associated to kidney biopsies are those related to bleeding, including hematomas and gross hematuria<sup>[87]</sup>. Iversen and Brun, in 1951 reported the first large series of needle biopsies in kidneys<sup>[88]</sup>. Later on, Parrish *et al*<sup>[89]</sup> also engaged in the labor of performing renal biopsies. Initially, the position of the kidney was determined by abdominal X-ray. Later on, sonography became available. They reported the common complications encountered in a period of 37 years (1951-1988). Complications occurred in 7% of the total



**Figure 1** Diabetic nephropathy class. A: Glomerular basement membrane thickening; B: Mild mesangial expansion; C: Severe mesangial expansion; D: Nodular sclerosis (Kimmelstiel-Wilson lesions); E: Advanced diabetic glomerulosclerosis.

**Table 2 Comparison of diabetic nephropathy and non-diabetic renal disease prevalence reported in the literature**

Ref.	Country	Population	Type 1 or 2 DM	% DN	% NDRD	% Mixed
Mazzucco <i>et al</i> <sup>[22]</sup>	Italy	393	2	39.7	43	17.3
Christensen <i>et al</i> <sup>[24]</sup>	Denmark	51	2	68.6	13.8	NR
Zhang <i>et al</i> <sup>[37]</sup>	China	130	2	73.9	26.1	NR
Zhuo <i>et al</i> <sup>[50]</sup>	China/Japan	216	2	6.5	82.9	10.7
Sharma <i>et al</i> <sup>[51]</sup>	United States	620	2	37	36	27
Hironaka <i>et al</i> <sup>[52]</sup>	Japan	35	1 and 2	71.4	14.3	14.3
Wong <i>et al</i> <sup>[53]</sup>	China	68	2	35	46	19
Prakash <i>et al</i> <sup>[55]</sup>	India	23	2	56.5	30.5	13
Mak <i>et al</i> <sup>[56]</sup>	China	51	2	67	16	17
Biensebach <i>et al</i> <sup>[57]</sup>	Austria	84	2	78.5	21.5	NR
Richards <i>et al</i> <sup>[58]</sup>	United Kingdom	68	1 and 2	61	32	3
Parving <i>et al</i> <sup>[59]</sup>	Denmark	35	2	77.1	20	2.9
Cordonnier <i>et al</i> <sup>[60]</sup>	United Kingdom	26	2	85	15	NR
Nzerue <i>et al</i> <sup>[61]</sup>	United States	31	2	41.9	19.4	38.7
Lee <i>et al</i> <sup>[62]</sup>	South Korea	22	2	36.4	50	13.6
Izzedine <i>et al</i> <sup>[63]</sup>	France	21	1 and 2	62	38	NR
Castellano <i>et al</i> <sup>[64]</sup>	Spain	20	2	45	55	NR
Serra <i>et al</i> <sup>[65]</sup>	Spain	35	2	74.3	17.2	8.5
Premalatha <i>et al</i> <sup>[66]</sup>	India	18	2	50	50	NR
Rychlík <i>et al</i> <sup>[67]</sup>	Czech Republic	163	2	42.4	47.5	10.1
Tone <i>et al</i> <sup>[68]</sup>	Japan	97	2	36	16.5	47.5
Moger <i>et al</i> <sup>[69]</sup>	India	26	2	34.6	23.1	42.3
Soni <i>et al</i> <sup>[70]</sup>	India	160	2	42.5	27.5	30
Pham <i>et al</i> <sup>[71]</sup>	United States	233	2	27.5	53.2	19.3
Kharrat <i>et al</i> <sup>[72]</sup>	Tunisia	72	2	34.1	69.5	NR
Akimoto <i>et al</i> <sup>[73]</sup>	Japan	50	2	68	26	6
Huang <i>et al</i> <sup>[74]</sup>	China	52	2	55.7	38.5	5.8
Lin <i>et al</i> <sup>[75]</sup>	Taiwan, China	50	2	48	22	30
Ghani <i>et al</i> <sup>[76]</sup>	Kuwait	31	2	54.8	NR	45.2
Arif <i>et al</i> <sup>[77]</sup>	Pakistan	73	2	27.3	49.3	NR
Hashim Al-Saedi <i>et al</i> <sup>[78]</sup>	Iraq	80	1 and 2	NR	NR	100
Mou <i>et al</i> <sup>[79]</sup>	China	69	2	47.8	52.2	NR
Haider <i>et al</i> <sup>[80]</sup>	Austria	567	1 and 2	68	17.4	NR
Chang <i>et al</i> <sup>[81]</sup>	South Korea	119	2	36.2	53.8	10
Bi <i>et al</i> <sup>[82]</sup>	China	220	2	54.5	NR	45.5
Chong <i>et al</i> <sup>[83]</sup>	Malaysia	110	2	62.7	18.2	19.1
Harada <i>et al</i> <sup>[84]</sup>	Japan	55	2	54.5	34.5	10.9
Oh <i>et al</i> <sup>[85]</sup>	South Korea	126	2	39.7	51.6	8.7
Yaqub <i>et al</i> <sup>[86]</sup>	Pakistan	68	2	31	52	17

DN: Diabetic nephropathy; NDRD: Non-diabetic renal disease; DM: Diabetes mellitus.

biopsies performed (> 1800), consisting mainly of gross hematuria lasting for more than 12 h and pain.

With the introduction of the ultrasound, renal biopsy has become easier and safer. Ultrasound-guided biopsy is the standard method to obtain kidney tissue for diagnosis<sup>[90]</sup>. Currently, complications are usually minor<sup>[37]</sup>. A recent meta-analysis that included more than 9400 renal biopsies showed a small risk of macroscopic hematuria of 3%, only requiring blood transfusion in 0.9% of the cases<sup>[91]</sup>. However, these events are not considered to represent serious medical problems; as they resolve within few hours after the procedure. Bleeding risks are also reduced by using smaller needle gauge, in order to obtain less tissue; but with adequate number of glomeruli per biopsy specimen for pathological diagnosis<sup>[90]</sup>.

Major complications, such as embolization of the renal artery, surgical intervention or death are relatively low. Patients with higher serum creatinine levels, especially women, have shown higher complication rates<sup>[87,91]</sup>.

Biopsy should be avoided in patients with bleeding

problems, uncontrolled hypertension, or those unable to cooperate; as these cases have been associated with an increased risk for complications after renal biopsy<sup>[92]</sup>.

Relative contraindications include: severe azotemia, anatomic abnormalities of the kidney such as arterial aneurysm, anticoagulant use, pregnancy, and urinary tract infection<sup>[93]</sup>.

### Ongoing development of minimally invasive diagnostic tools

Some useful clinical indicators for DN are the presence of diabetic retinopathy and longer duration of diabetes. In contrast; for NDRD, signs include the presence of acute renal failure and microscopic hematuria<sup>[83]</sup>. However, these clinical markers are not completely accurate and therefore, efforts have been directed to develop more modern technology in non-invasive diagnosis of DN to help clinicians to decide when a kidney biopsy should be warranted. These include the use of imaging techniques as well as the identification of serum and urinary bio-

markers.

Nowadays, diagnostic imaging technology has evolved to help clinicians on their daily decision making regarding which patients to biopsy in order to confirm DN. Insalaco *et al.*<sup>[94]</sup> had reported the use of eco-colour-Doppler sampling of interlobular renal arteries and determination of their intrarenal resistance indices (RI) to differentiate DN from NDRD.

RI helps to measure hemodynamic changes in the renal arteries. These are usually seen in patients with DN, due to alterations in the compliance of the vessels affecting the blood flow. Therefore, early changes in blood flow are detected by renal Doppler and they may reflect the progression of DN<sup>[95]</sup>. RI higher than 0.70 is a strong predictor of disease progression to renal failure, as well as RI lower than 0.70 is associated to a slow progression of renal disease<sup>[96]</sup>. Also, it has been shown that RI in patients with DN is significantly higher than those with NDRD. RI evaluation could help determine prognosis and guide therapy; as this could potentially help to predict which patients with diabetes presenting with proteinuria should undergo renal biopsy; consequently reducing the indications for this procedure.

However, there is still no general agreement for the routine use of Doppler ultrasonography in patients with DN. Results may vary due to other factors that also modify renal vascular resistance; such as age, vascular compliance, high blood pressure, elevated heart rate, and the use of ACE-Inhibitors<sup>[97]</sup>.

Several serum circulating biomarkers may also help to identify patients that will develop DN in patients with diabetes and/or to identify those patients at risk to progress to ESRD in those with DN. Among them: uric acid<sup>[98]</sup>, vitamin D<sup>[99]</sup>, FGF23<sup>[100]</sup> and TNFR1 and TNFR2<sup>[101,102]</sup> are promising biomarkers.

Elevated serum concentrations of TNFR1 and TNFR2 are strongly associated with early renal function loss in patients with T1DM and T2DM<sup>[101,102]</sup>. In contrast, low complement levels (C3 and/or C4) and M-spike have been associated with NDRD (alone or with coexistent DN) in kidney biopsies<sup>[51]</sup>. Whether any of this biomarker could be causative of the disease initiation and progression remains to be proven through either experimental studies or through intervention studies. It would also be interesting to know if and how these biomarkers correlate to any given histological finding.

MicroRNA profiling has also been studied as a promising tool in the diagnosis of DN. Studies have been reported using this approach to determine different stages of diabetic nephropathy by analyzing urinary microRNA. This includes the potential benefit to distinguish early indicators of DN and to provide a tool for personalized medical therapy<sup>[103]</sup>.

Metabolomics is an evolving field dedicated to identify new metabolites predicting DN in patients with diabetes<sup>[104]</sup>. Similarly, analysis of urinary proteomics and urinary exosomes have yield promising results<sup>[105]</sup>. More recently, we have reported that a cell based assay where

normal human podocytes are cultured in the presence of the sera of patients with diabetes may help predict the progression to CKD<sup>[106]</sup>.

Finally, it would be interesting to have an integrative approach, where clinical phenotype combines to findings on kidney biopsies. This biologic application would likely represent a very powerful individualized diagnostic and prognostic tool in DN<sup>[107]</sup>.

## CONCLUSION

Unfortunately, patients with NDRD are often designated as having DN because of the overlapping features of glomerulopathies<sup>[70]</sup>. It is important to identify and differentiate these pathologies at an early stage in order to prevent progression and potential complications.

There is an overwhelming number of cases where these diagnoses would lead to changes in treatment, ranging from the use of immunosuppression to titration of renin-angiotensin-aldosterone system blockade<sup>[108]</sup>.

Common clinical practice is to biopsy patients with diabetes with a low pre-test probability for DN, such as patients presenting with AKI, low complements, and hematuria suggesting an increased likelihood of finding NDRD on biopsy<sup>[51]</sup>. Nevertheless, as loss of renal function correlates with increased mortality. Therefore, any intervention that would help to delay progression to ESRD should significantly increase survival. In view of the fact that histology is necessary to characterize different glomerular diseases originating several nonspecific clinical presentations, kidney biopsy would help to direct a better management<sup>[109]</sup>.

Routine use of renal biopsy should be implemented, especially in those with atypical features<sup>[83]</sup>, for several reasons: (1) it helps to characterize the epidemiological features of renal diseases in diabetic patients<sup>[110,111]</sup>; (2) it provides the opportunity to determine how histological and high-throughput profiling correlate with the clinical phenotype<sup>[111]</sup>; and (3) it set the basis for personalized management strategies<sup>[23,112]</sup>.

The ability to differentiate between renal pathologies other than DN (that could be reversed with a specific treatment) and DN would be of extreme importance. We strongly support the recommendation that kidney biopsy should become a routine tool in specific patients at high risk of developing CKD, especially in those cases where this practice helps to reverse and/or prevent further kidney damage as it will help to direct assertive and aggressive treatment to many of the non-diabetic nephropathies to prevent or delay poor outcomes<sup>[81,108]</sup>.

There is lack of studies in the literature regarding the universal use of kidney biopsy on patients with diabetes. As new studies have become available to demonstrate how quantitative histological features may predict the disease course earlier than albuminuria<sup>[113]</sup>, our level of confidence to perform routine kidney biopsies in patients with diabetes should increase. New research studies are required, longitudinal observational clinical trials

as well as interventional trials, where the implementation of routine kidney biopsy is evaluated for patients with diabetes at time of diagnosis to evidence improvement in outcomes. These findings on kidney biopsies may help select the population of patients at highest risk of disease progression and may offer a new hard outcome measure to study DN.

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## Prescribing physical activity to prevent and manage gestational diabetes

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### Abstract

Gestational diabetes mellitus (GDM) is the most prevalent metabolic disorder during pregnancy. Women diagnosed with GDM have a substantially greater risk of developing type 2 diabetes within 5-10 years after delivery, and the risk is increased by excess body weight. Uncontrolled hyperglycemia during pregnancy is potentially harmful to both mother and fetus, resulting in a greater need for Caesarian-section deliveries, delivery of larger infants with more excess body fat, a greater risk of infant death and stillbirth, and an elevated risk of infant hypoglycemia immediately after birth. Fortunately, engaging in physical activity prior to and during pregnancy may lower the risk of developing GDM. Pregnant women should also be advised how to safely increase their physical activity during pregnancy and the postpartum period. An initial approach to becoming more physically active can simply be to encourage women to incorporate more unstructured physical activity into daily living, both before and during pregnancy. Giving women an appropriate exercise prescription can encourage them to participate in physical activity

safely and effectively throughout pregnancy to prevent and/or manage GDM. Engaging in 30 min of moderate intensity physical activity on most, if not all, days of the week has been adopted as a recommendation for all pregnant women.

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**Key words:** Gestational diabetes; Pregnancy; Physical activity; Exercise prescription; Blood glucose

**Core tip:** Gestational diabetes mellitus is the most prevalent metabolic disorder during pregnancy. Uncontrolled hyperglycemia during pregnancy is potentially harmful to both mother and fetus. Pregnant women should be advised how to safely increase their physical activity during pregnancy and the postpartum period. Giving women an appropriate exercise prescription can encourage them to participate in physical activity safely and effectively throughout pregnancy to prevent and/or manage gestational diabetes. Engaging in 30 min of moderate intensity physical activity on most, if not all, days of the week has been adopted as a recommendation for all pregnant women.

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### INTRODUCTION

Gestational diabetes mellitus (GDM) is the most prevalent metabolic disorder during pregnancy. Defined as glucose intolerance of varying severity diagnosed during pregnancy that usually resolves postpartum, it typically

occurs during the middle of pregnancy and is diagnosed at 24 to 28 wk of gestation with an oral glucose tolerance test<sup>[1,2]</sup>. Currently, gestational diabetes affects 18 percent of pregnancies in the United States and has been increasing in prevalence<sup>[2]</sup>.

Women diagnosed with GDM have a substantially greater risk of developing type 2 diabetes (T2D) within 5-10 years after delivery, and the risk is increased by excess body weight<sup>[2,3]</sup>. Uncontrolled hyperglycemia during pregnancy is potentially harmful to both mother and fetus, resulting in a greater need for Caesarian-section deliveries, delivery of larger infants with more excess body fat, a greater risk of infant death and stillbirth, and an elevated risk of infant hypoglycemia immediately after birth<sup>[4,5]</sup>.

Sufficient evidence supports the promotion of moderate-to-vigorous prenatal physical activity for maternal health benefits<sup>[6]</sup>, and physical activity prior to and during pregnancy may lower the risk of developing GDM<sup>[7-11]</sup>. When prescribed effectively, physical activity can also be undertaken safely by nearly all pregnant women.

## ETIOLOGY OF GESTATIONAL DIABETES

GDM can be considered as a transient form of T2D, with its rapid onset triggered by the metabolic and hormonal changes of pregnancy. Normal pregnancies are associated with increased insulin resistance, which begins in mid-pregnancy and continues until delivery. This resistance is usually compensated by a 200 to 250 percent increase in insulin secretion during pregnancy<sup>[12]</sup>. The pregnant woman's placenta releases hormones that block the effect of circulating insulin and making the mother more insulin resistant in order to nurture the growing fetus. While placental growth hormone induces maternal insulin resistance and mobilizes maternal nutrients for fetal growth, human placental lactogen and prolactin increase maternal food intake by inducing central leptin resistance and promoting maternal  $\beta$ -cell expansion and insulin production<sup>[13]</sup>. As a result, the mother's insulin needs to increase as much as three or more times normal during pregnancy, resulting in hyperglycemia if her pancreatic  $\beta$ -cells are unable to keep up with heightened insulin demands. A higher body fat percentage, physical inactivity, and diet quality are important modifiable risk factors for GDM<sup>[14]</sup>.

Given that a sedentary lifestyle can contribute to its development, physical activity prior to and during pregnancy may lower the risk of developing GDM. Although few studies have focused on the mechanisms of exercise-induced benefits in GDM, due to the similarity between GDM and T2D, most of the suggested mechanisms in diabetes can be extrapolated to GDM. The benefits of physical activity to prevent and treat GDM are associated with increased insulin sensitivity, beneficial adipokines changes, and reduced oxidative stress related to the antioxidant effect of exercise<sup>[15]</sup>.

## OVERVIEW OF THE DIAGNOSIS AND TREATMENT OF GESTATIONAL DIABETES

There is a current controversy on the most appropriate diagnostic test(s) for GDM. It is paramount that health care practitioners understand that any degree of abnormal glucose homeostasis in pregnancy independently predicts an increased risk of glucose intolerance postpartum<sup>[16]</sup>, and women with either GDM or gestational impaired glucose tolerance exhibit declining  $\beta$ -cell function in the first year after giving birth that likely contributes to their future diabetes risk<sup>[17]</sup>. Women with a history of GDM should have lifelong screening for T2D or pre-diabetes at least every 3 years<sup>[1]</sup>.

If maternal hyperglycemia is not controlled, the elevated blood glucose levels are elevated for both the mother and the developing fetus and can lead to macrosomia (a baby weighing more than is appropriate for his or her gestational age). Macrosomia usually occurs as the result of an excessive availability of nutrients and an increase in fetal insulin release<sup>[18]</sup>. Such babies can face health problems, including damage to their shoulders during birth, low blood glucose levels following birth, and acute breathing problems. In addition, babies with excess fat and elevated insulin levels are at higher risk for obesity and T2D later in life.

Therefore, the primary goal of treatment of GDM is to keep blood glucose levels within normal limits throughout the pregnancy to ensure appropriate fetal growth. Implementation of lifestyle changes (*i.e.*, diet and exercise) to manage glycemic control is the first management strategy employed<sup>[19-22]</sup>. A recommended diet for women with GDM is moderate in fat and protein and provides controlled levels of carbohydrates<sup>[23]</sup>. Women should be advised to avoid or limit their intake of refined carbohydrates, sugary drinks, fruit juices, pastries, and other sweets that require large amount of insulin to manage blood glucose levels after ingestion. Pregnant women generally will require no more than 300 extra calories daily to cover their increased energy requirements which they are often already consuming prior to pregnancy<sup>[19]</sup>.

Pregnant women should also be advised how to safely increase their physical activity during pregnancy and the postpartum period. This is often an overlooked tool to help normalize blood glucose levels and may prevent or delay the need for insulin. Physical activity performed during and after pregnancy benefits a woman's overall health<sup>[24]</sup>. Instead of detraining, pregnant women undertaking moderate or higher intensity physical activity can maintain or increase cardiorespiratory fitness<sup>[25]</sup>. Maternal exercise during pregnancy does not increase the risk of low birth weight, preterm delivery, or early pregnancy loss; in fact, exercise undertaken on a regular basis may reduce the risk of pregnancy complications, such as

preeclampsia and GDM, and shortens the duration of active labor<sup>[7,8,26-29]</sup>. Physical activity is not associated with risks for the newborn and can lead to changes in lifestyle that imply long-term benefits<sup>[30]</sup>.

If lifestyle changes are not successful in maintaining target glucose values during pregnancy, glucose-lowering medications may additionally be used<sup>[31-33]</sup>. Insulin remains the gold standard for pharmacologic therapy and is still recommended by many practitioners over oral hypoglycemic agents<sup>[1]</sup>. Both glyburide and metformin have been shown to cross the placenta to the fetus and should be used with caution and patients counseled appropriately<sup>[31]</sup>. Regular aerobic training may result in a lowering of the daily insulin dose needed to manage glucose levels in some women with GDM, which often feels like a reward to patients.

## PREVENTION OF GESTATIONAL DIABETES WITH PHYSICAL ACTIVITY

Engaging in regular physical activity before pregnancy frequently has been associated with a reduced risk of developing GDM<sup>[7,8,26-29]</sup>. A prospective cohort study among 21765 women in the Nurses' Health Study II showed that physical activity before pregnancy is associated with a risk reduction in GDM, and both intense exercise and moderate activity (*e.g.*, brisk walking) bestow a similar risk reduction<sup>[28]</sup>. Even engaging in leisure time physical activity in advance of becoming pregnant may reduce glucose intolerance during the pregnancy<sup>[34,35]</sup>.

Being physically active during pregnancy may prevent both GDM and possibly later-onset T2D<sup>[56]</sup>, but studies have shown mixed results<sup>[7,26,27]</sup>. Women who perform recreational physical activity during the year before becoming pregnant experience a reduced risk, but participating in any physical activity during the first 20 wk of pregnancy has been shown to lead to close to a 50 percent risk reduction in GDM<sup>[7]</sup>. Engaging in physical activity both before and during pregnancy likely leads to the greatest reduction in GDM risk<sup>[7]</sup>.

Exogenous insulin requirements can be lowered and glycemic control improved after a four- to eight-week period of exercise in the last half of pregnancy, even when exercise is milder in terms of duration and frequency<sup>[37]</sup>. In women with GDM unresponsive to dietary changes, eight weeks of moderate, supervised exercise undertaken three times a week maintained blood glucose levels within normal limits without the need for exogenous insulin<sup>[38]</sup>. However, exercise must be performed on a chronic basis to have a sustained impact on glycemic control.

When compared with less vigorous activities, an exercise intensity that reaches at least 60 percent of heart rate reserve (HRR) during pregnancy, while gradually increasing physical activity energy expenditure, reduces the risk of developing GDM<sup>[22]</sup>. The more vigorous the exercise, the less total exercise time is required. In a recent clinical trial, a moderate physical activity program performed thrice weekly during pregnancy improved

levels of maternal glucose tolerance in healthy, pregnant women<sup>[39]</sup>, and higher levels of physical activity participation before pregnancy or in early pregnancy significantly lowered the risk of developing GDM<sup>[10]</sup>. Moreover, a recent study reported that women can safely engage in aerobic exercise and resistance training for muscular endurance 3 d per week for 30 min throughout gestation and doing so reduces the incidence of GDM<sup>[11]</sup>. Thus, it appears that higher levels of moderate physical activity (aerobic or resistance training) may reduce the risk of developing GDM during pregnancy and lower blood glucose levels in women who do develop it.

To achieve the best decline in glucose concentrations, pregnant women may need to walk for 25 min per session at a vigorous intensity or for 35 to 40 min per session at low intensity if they are at risk for GDM and for at least 25 min at either low or vigorous intensity if they have a low risk to achieve optimal benefits related to glycemic control and prevention of GDM<sup>[9]</sup>.

Not all studies have shown that physical activity can prevent the onset of GDM. For example, a recent review of five randomized controlled trials in 2012 reported limited evidence available on the effect of exercise during pregnancy for preventing pregnancy-induced hyperglycemia, although at least seven additional trials are currently underway<sup>[40]</sup>. Similarly, a 12-wk exercise program (30-35 min low impact aerobics, 20-25 min of strength exercises using body weight as resistance and 5-10 min of stretching, breathing, and relaxation exercises) undertaken during the second trimester of pregnancy did not reduce the prevalence of gestational diabetes in pregnant women with a body mass index in the normal range<sup>[41]</sup>. In a recent study involving moderate-intensity resistance and aerobic exercises (done three times per week for 50 to 55 min per session), the risk of developing GDM also was not lowered<sup>[42]</sup>. Larger, well-designed randomized trials, with standardized behavioral interventions are definitely needed to assess the effects of exercise on preventing GDM or other adverse pregnancy outcomes.

## MANAGEMENT OF GESTATIONAL DIABETES WITH PHYSICAL ACTIVITY

Women with diagnosed GDM can experience greater blood glucose uptake through increased insulin sensitivity from both aerobic and resistance training<sup>[43,44]</sup>. A meta-analysis reported that pregnant women with GDM who exercised on a cycle or arm ergometer or performed resistance training three times a week for 20 to 45 min experience better glycemic control, lower fasting and postprandial glucose levels, and improve cardiorespiratory fitness<sup>[25]</sup>. Nevertheless, being active doing any type of activity did not necessarily prevent the need for supplemental insulin to manage blood glucose levels or change pregnancy outcomes.

On the other hand, at least one study reported that resistance exercise training may help to avoid insulin therapy for overweight women with GDM<sup>[44]</sup>. In addi-

**Table 1** Contraindications to physical activity during pregnancy and warning signs for termination

Contraindications to exercise <sup>[19]</sup>	Significant cardiopulmonary disease, restrictive lung disease, incompetent cervix/cerclage, multiple gestation at risk for premature labor, persistent second- or third-trimester bleeding, placenta previa after 26 wk of gestation, premature labor during the current pregnancy, ruptured membranes, and preeclampsia/pregnancy-induced hypertension
Warning signs to terminate or avoid exercise	Vaginal bleeding, dyspnea prior to exertion, dizziness, headache, chest pain, muscle weakness, calf pain or swelling, preterm labor, decreased fetal movement, or amniotic fluid leakage

tion, regular physical activity may improve other pregnancy outcomes, such as lowering the GDM-related risk of having a newborn with macrosomia by 58 percent, and the risk of having acute elective caesarean delivery by 34 percent<sup>[42]</sup>. The intensity of activities undertaken may also impact glycemic management. For example, a low-intensity walking program has been shown to lower capillary blood glucose measurements in women with GDM<sup>[20]</sup>; however, a higher intensity of exercise may provide additional benefits.

## EXERCISE PRESCRIPTION FOR WOMEN WITH GESTATIONAL DIABETES

An initial approach to helping women become more physically active can simply be to encourage them to incorporate more unstructured physical activity into daily living, both before and during pregnancy<sup>[45]</sup>. While such lifestyle activity does not entirely take the place of traditional structured exercise, it can be highly effective in preventing excessive weight gain, increasing daily activity levels, and building a fitness base from which to progress to prescribed exercise, as follows.

Unless a woman has medical reasons to avoid physical activity during pregnancy, such as premature labor, significant cardiopulmonary disease, or preeclampsia, she can begin or continue moderate-intensity aerobic physical activity during her pregnancy and after giving birth to help manage her blood glucose levels<sup>[10,14,20,21,23,25,43,44,46]</sup>. Warning signs to terminate exercise while pregnant include vaginal bleeding, dyspnea prior to exertion, dizziness, headache, chest pain, muscle weakness, calf pain or swelling, preterm labor, decreased fetal movement or amniotic fluid leakage<sup>[19]</sup>. Contraindications to and warning signs to end physical activity are listed in Table 1.

### Mode of physical activity

Most pregnant women with and without GDM can safely undertake moderate and vigorous aerobic activities. These include both weight-bearing activities like walking, jogging, running, conditioning machines (*e.g.*, elliptical trainers), and dancing and non-weight-bearing ones like, cycling, swimming, water aerobics, aquatic activities, chair exercises, and rowing<sup>[19]</sup>.

Resistance training can also be safely and effectively undertaken by pregnant women with GDM and may reduce the need for exogenous insulin to control hyperglycemia<sup>[21]</sup>. The most recent guidelines propose increasing weekly physical-activity expenditure while incorporating

vigorous exercise and adding light strength training to the exercise routine of healthy pregnant women<sup>[22]</sup>. Most of these exercises can be completed either standing or sitting, as desired for comfort and safety. Low- to moderate-intensity muscle-strengthening exercises performed during the second and third trimesters of pregnancy have a minimal effect on newborn body size and overall health<sup>[22,47]</sup>. Thus, women with GDM can experience greater blood glucose uptake through increased insulin sensitivity from both aerobic and resistance training<sup>[43,44]</sup>.

During pregnancy, however, women should avoid doing exercises involving lying on their backs during the second and third trimesters. They should also avoid activities that increase the risk of falling or abdominal trauma, including contact or collision sports, horseback riding, downhill skiing, water skiing, soccer, and basketball. Late in pregnancy, non-weight-bearing activities may be preferable to weight-bearing activities in some women, especially if low back pain is present<sup>[48]</sup>.

### Intensity of physical activity

For most healthy women who are not already highly active or doing vigorous-intensity activity, moderate-intensity aerobic activity is recommended during pregnancy and the postpartum period, corresponding to 40%-59% HRR, "somewhat hard," or 5-6 on a 10-point rating scale<sup>[49,50]</sup>. A more deconditioned woman may start as low as 30% HRR and progress to moderate levels. Women who are already highly active or doing regular vigorous activity (60%-89% HRR, "hard," or a rating of 7-8) can continue these activities during pregnancy.

Because the effects of vigorous-intensity aerobic activity during pregnancy have not been studied carefully, there is no basis for recommending that women should begin such activities during pregnancy if they already were not doing so. Women who habitually engage in vigorous or high amounts of activity or strength training should continue to be physically active during pregnancy and after giving birth; they generally do not need to drastically reduce their activity levels, provided that they remain healthy and discuss with their health-care provider how to adjust activity levels during this time<sup>[19]</sup> (Table 2).

### Frequency of physical activity

According to current guidelines, pregnant women should engage in physical activity on most, if not all, days of the week, including both aerobic and resistance training<sup>[19,22,47]</sup>. Current guidelines for adults generally recommend five sessions of moderate activity, which would

**Table 2** Example using heart rate reserve to determine target hazard ratio range

Target HR range (40%-89% HRR)
Example patient: 30-year-old female
Resting HR: 78 beats per minute (bpm)
Maximum HR: 190 bpm (estimated as 220 minus age)
Formula for sample calculation:
Target HR = [(Max HR-Resting HR) × Desired Intensity] + Resting HR
Lower end of HR range (40%) = [(190-78) × 0.40] + 78
(Low end of moderate intensity) = [112 × 0.40] + 78
= 45 + 78
= 123 bpm
Higher end of HR range (89%) = [(190-78) × 0.85] + 78
(High end of vigorous intensity) = [112 × 0.89] + 78
= 100 + 78
= 178 bpm
Target HR range (40%-89% HRR) = 123 to 178 bpm

HRR: Heart rate reserve.

also apply to women with GDM<sup>[49,50]</sup>. Daily exercise may enhance glucose metabolism further and, therefore, the recommended frequency for any type of physical activity for women with GDM is a minimum of three and up to seven days per week, spread throughout the week<sup>[19]</sup>.

### Duration of physical activity

Engaging in 30 min of moderate intensity physical activity on most days of the week, reaching a minimal total of 150 min per week, has been adopted as a recommendation for pregnant women without medical or obstetrical complications<sup>[19]</sup>. Health benefits can be derived from daily sessions lasting 20 to 45 min<sup>[25]</sup>. Compared with less vigorous activities, engaging in an exercise intensity that is at least 60 percent of HRR during pregnancy, while gradually increasing physical activity energy expenditure, reduces the risk of GDM, and the more vigorous the exercise, the less total exercise time is required. Prolonged duration physical activity (*i.e.*, lasting over 60 to 90 min when done continuously) usually is not recommended for pregnant women due to heightened concern over possible hypoglycemia or hyperthermia, however<sup>[29]</sup>.

### Progression of physical activity

Sedentary and deconditioned women with GDM should start out on the low end of the intensity scale (30%-39% HRR) and gradually progress to moderate-intensity exercise (40%-59% HRR) or higher. Initially, they should be advised to increase frequency and duration of activities rather than intensity. For previously inactive women, moderate-intensity workouts are an appropriate endpoint, but if just starting physical activity during pregnancy, women should progress gradually over time to that intensity. Women who have been active before and during pregnancy and before diagnosis of GDM should continue doing moderate-to-vigorous intensity activities<sup>[47]</sup>.

During a normal postpartum period, regular physical activity continues to benefit a woman's overall health. Moderate-intensity physical activity undertaken after giv-

**Table 3** Recommended exercise prescription for women with gestational diabetes

Mode	Aerobic: Walk, stationary cycle, swim, aquatic activities, conditioning machines, prenatal exercise classes, prenatal yoga, seated exercises, and possibly jogging or running (if highly active before pregnancy) Resistance: Light or moderate resistance exercises Exercises to Avoid: Activities lying flat on the back and any that increase the risk of falling or abdominal trauma ( <i>e.g.</i> , contact or collision sports, horseback riding, downhill skiing, water skiing, soccer, outdoor cycling, basketball, most racquet sports, and scuba diving)
Intensity	If inactive: moderate-intensity aerobic activity (40%-59% HRR, or "somewhat hard") during pregnancy and postpartum If already active or doing vigorous activity: moderate- to vigorous-intensity activity (40%-89% HRR, or "somewhat hard" to "hard")
Frequency	3-7 d, spread throughout the week Better done on most, if not all, days of the week
Duration	30 min/session (range of 20-45 min) At least 150 min of moderate-intensity physical activity spread throughout the week
Progression	If just starting, increase duration of moderate exercise slowly; if already more active, maintain or lower intensity during pregnancy rather than attempting to progress to higher levels

HRR: Heart rate reserve. Reprinted by permission from (Colberg SR. Exercise and Diabetes: A Clinician's Guide to Prescribing Physical Activity, American Diabetes Association, Alexandria, VA, United States, 2013. p. 110.).

ing birth increases cardiorespiratory fitness and improves mood, with no adverse effects on breast milk volume, breast milk composition, or infant growth<sup>[51]</sup>. An added benefit is that it helps women achieve and maintain a healthy weight postpartum and can promote weight loss when combined with caloric restriction. Pregnant women who habitually engage in vigorous-intensity aerobic activity or are highly active can continue such physical activity during pregnancy and the postpartum period, provided that they remain healthy and avoid activities that increase risk of falls and traumatic injury (Table 3).

## CONCLUSION

In conclusion, women at high risk for GDM may be able to prevent it with lifestyle management before and during pregnancy. In those who develop GDM, dietary improvements and regular physical activity are frequently sufficient to manage hyperglycemia, although insulin may be used when these changes are not enough. Management of blood glucose levels ensures better pregnancy outcomes and improves the health of both the mother and the fetus. Giving pregnant women an appropriate exercise prescription can encourage them to participate in physical activity safely and effectively throughout pregnancy to prevent and/or manage gestational diabetes. Engaging in 30 min of moderate intensity physical activity on most, if not all, days of the week has been adopted as a recommendation for all pregnant women.

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WJD 5<sup>th</sup> Anniversary Special Issues (1): Insulin**Down-regulation of pancreatic transcription factors and incretin receptors in type 2 diabetes**

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**Core tip:** Expression of pancreatic transcription factors and incretin receptors is decreased in diabetes.

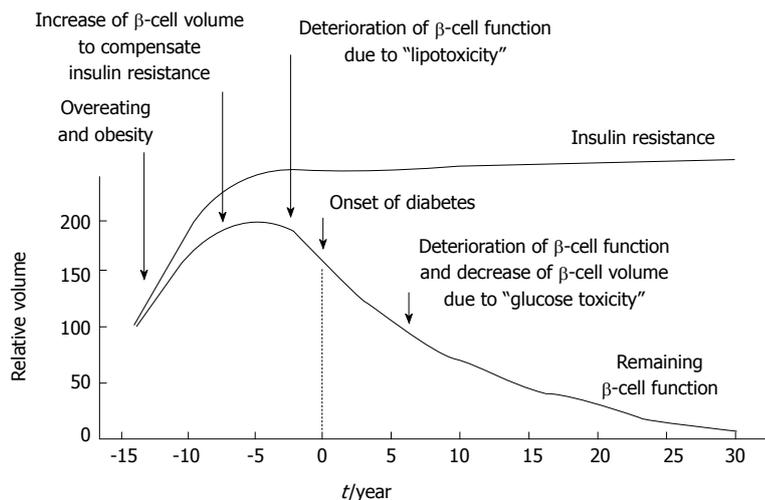
Kaneto H, Matsuoka TA. Down-regulation of pancreatic transcription factors and incretin receptors in type 2 diabetes. *World J Diabetes* 2013; 4(6): 263-269 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/263.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.263>

**Abstract**

Type 2 diabetes is one of the most prevalent and serious metabolic diseases. Under diabetic conditions, chronic hyperglycemia and subsequent induction of oxidative stress deteriorate pancreatic  $\beta$ -cell function, which leads to the aggravation of type 2 diabetes. Although such phenomena are well known as glucose toxicity, its molecular mechanism remains unclear. In this review article, we describe the possible molecular mechanism for  $\beta$ -cell dysfunction found in type 2 diabetes, focusing on (1) oxidative stress, (2) pancreatic transcription factors (PDX-1 and MafA) and (3) incretin receptors (GLP-1 and GIP receptors). Under such conditions, nuclear expression levels of PDX-1 and MafA are decreased, which leads to suppression of insulin biosynthesis and secretion. In addition, expression levels of GLP-1 and GIP receptors are decreased, which likely contributes to the impaired incretin effects found in diabetes. Taken together, it is likely that down-regulation of pancreatic transcription factors (PDX-1 and MafA) and down-regulation of incretin receptors (GLP-1 and GIP receptors) explain, at least in part, the molecular mechanism for  $\beta$ -cell dysfunction found in type 2 diabetes.

**INVOLVEMENT OF OXIDATIVE STRESS IN THE DETERIORATION OF  $\beta$ -CELL FUNCTION FOUND IN TYPE 2 DIABETES**

The development of type 2 diabetes is associated with pancreatic  $\beta$ -cell dysfunction and insulin resistance. First, overeating and/or obesity lead to the development of insulin resistance and normal  $\beta$ -cells secrete a larger amount of insulin to compensate for the increased insulin resistance. Next, large adipocytes secrete a larger amount of free fatty acids (FFAs) and/or various inflammatory cytokines which gradually deteriorate  $\beta$ -cell function and finally lead to the onset of diabetes. This process is known as “ $\beta$ -cell lipotoxicity”. Indeed, it has been reported that when islets or  $\beta$ -cell-derived cell line are exposed to FFAs, oxidative stress is induced, which leads to the reduction of insulin secretion<sup>[1-5]</sup>. It has also been reported that FFA-mediated induction of inducible nitric oxide synthase (iNOS) and excess nitric oxide (NO) generation are involved in the progression of  $\beta$ -cell



**Figure 1 Typical progress of type 2 diabetes.** The development of type 2 diabetes is associated with pancreatic  $\beta$ -cell dysfunction and insulin resistance. First, overeating and/or obesity lead to the development of insulin resistance and normal  $\beta$ -cells secrete a larger amount of insulin to compensate for the increased insulin resistance. Next, large adipocytes secrete a larger amount of free fatty acids and/or various inflammatory cytokines which gradually deteriorate  $\beta$ -cell function and finally lead to the onset of diabetes. This process is known as “ $\beta$ -cell lipotoxicity”. Once hyperglycemia becomes apparent,  $\beta$ -cell function progressively deteriorates; insulin biosynthesis and secretion are reduced. This process is known as “ $\beta$ -cell glucose toxicity” which is often observed in type 2 diabetes.

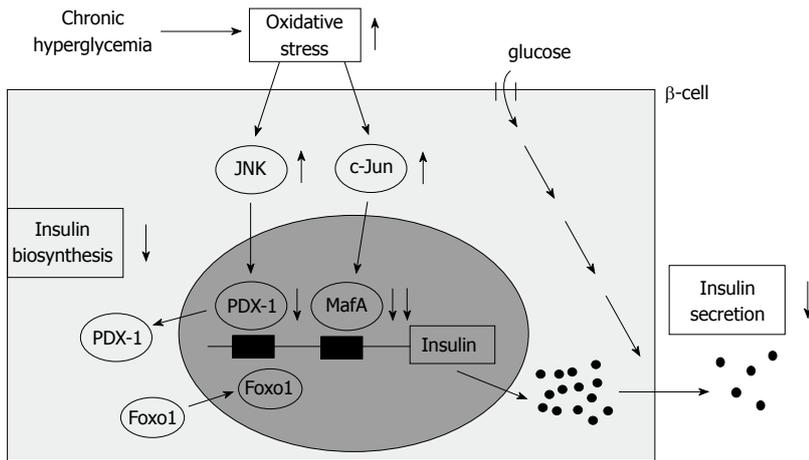
dysfunction<sup>[6]</sup>. Once hyperglycemia becomes apparent,  $\beta$ -cell function such as insulin biosynthesis and secretion progressively deteriorates. This process is known as “ $\beta$ -cell glucose toxicity” which is often observed under diabetic conditions. In the diabetic state, hyperglycemia per se and subsequent induction of oxidative stress decrease insulin biosynthesis and secretion and finally bring about apoptosis<sup>[7-28]</sup> (Figure 1).

Under diabetic conditions, oxidative stress is induced and involved in the  $\beta$ -cell glucose toxicity<sup>[22-36]</sup>.  $\beta$ -cells express GLUT2, a high-Km glucose transporter, and thereby display highly efficient glucose uptake when exposed to a high glucose concentration. Indeed, it was shown that expression levels of oxidative stress markers such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxy-2, 3-nonenal (4-HNE) were increased in islets under diabetic conditions<sup>[14,16]</sup>. In addition,  $\beta$ -cells are rather vulnerable to oxidative stress due to the relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase. Therefore, it is likely that oxidative stress is involved in the deterioration of  $\beta$ -cell function found in diabetes. It was shown that when  $\beta$ -cell-derived cell lines or isolated islets were exposed to oxidative stress, insulin gene promoter activity and mRNA expression were suppressed<sup>[19-26]</sup>. In addition, when they were exposed to oxidative stress, bindings of pancreatic transcription factors PDX-1 and/or MafA to the insulin gene promoter were reduced. It is noted here that PDX-1 plays a crucial role in pancreas development,  $\beta$ -cell differentiation, induction of surrogate  $\beta$ -cells, and maintenance of mature  $\beta$ -cell function<sup>[29-41]</sup> and that MafA is a  $\beta$ -cell-specific transcription factor and functions as a potent activator of insulin gene transcription<sup>[42-47]</sup>. Furthermore, it was shown that the decrease of insulin gene expression after chronic exposure to a high glucose concentration was prevented by treatment with antioxidants<sup>[16,19,25,26]</sup>. Reduction of expression and/or DNA binding activities of PDX-1 and/or MafA by chronic exposure to high glucose was also prevented by an antioxidant treatment. These results suggest that chronic hyperglycemia suppresses insulin biosynthesis

and secretion by increasing oxidative stress, accompanied by reduction of expression and/or DNA binding activities of two important pancreatic transcription factors, PDX-1 and MafA. Therefore, it is likely that the alteration of such transcription factors explains, at least in part, the suppression of insulin biosynthesis and secretion, and thereby is involved in  $\beta$ -cell glucose toxicity (Figure 2).

### MOLECULAR MECHANISM FOR DOWN-REGULATION OF NUCLEAR PDX-1 EXPRESSION UNDER DIABETIC CONDITIONS: POSSIBLE INVOLVEMENT OF OXIDATIVE STRESS AND SUBSEQUENT ACTIVATION OF THE JNK PATHWAY

It has been suggested that activation of the c-Jun N-terminal kinase (JNK) pathway is involved in pancreatic  $\beta$ -cell dysfunction found in type 2 diabetes. It was reported that activation of the JNK pathway is involved in reduction of insulin gene expression by oxidative stress and that suppression of the JNK pathway can protect  $\beta$ -cells from oxidative stress<sup>[48]</sup>. When isolated islets were exposed to oxidative stress, the JNK pathway was activated, preceding the decrease of insulin gene expression. Adenoviral overexpression of dominant-negative type JNK1 (DN-JNK) protected insulin gene expression and secretion from oxidative stress. These results were correlated with change in the binding of PDX-1 to insulin gene promoter. Adenoviral overexpression of DN-JNK preserved PDX-1 DNA binding activity in the face of oxidative stress, while wild type JNK (WT-JNK) overexpression decreased PDX-1 DNA binding activity<sup>[48]</sup>. Taken together, it is likely that activation of the JNK pathway leads to decreased PDX-1 activity and consequent suppression of insulin gene transcription found in the diabetic state. Also, it was shown that PDX-1 was transported from the nuclei to the cytoplasm in response to oxidative stress. When  $\beta$ -cell-derived HIT cells were



**Figure 2** Possible molecular mechanism for suppression of insulin biosynthesis in type 2 diabetes. Under diabetic conditions, hyperglycemia induces oxidative stress and thereby leads to suppression of insulin biosynthesis and secretion which is accompanied by reduction of nuclear pancreatic duodenal homeobox 1 (PDX-1) and MafA expression. Oxidative stress and subsequent activation of the JNK pathway translocate Foxo1 from cytoplasm to nuclei, leading to translocation of PDX-1 from nuclei to cytoplasm in  $\beta$ -cells. In addition, oxidative stress and subsequent induction of c-Jun expression suppress nuclear expression of MafA in  $\beta$ -cells. Therefore, it is likely that induction of oxidative stress and suppression of nuclear PDX-1 and MafA expression are involved in  $\beta$ -cell glucose toxicity found in type 2 diabetes.

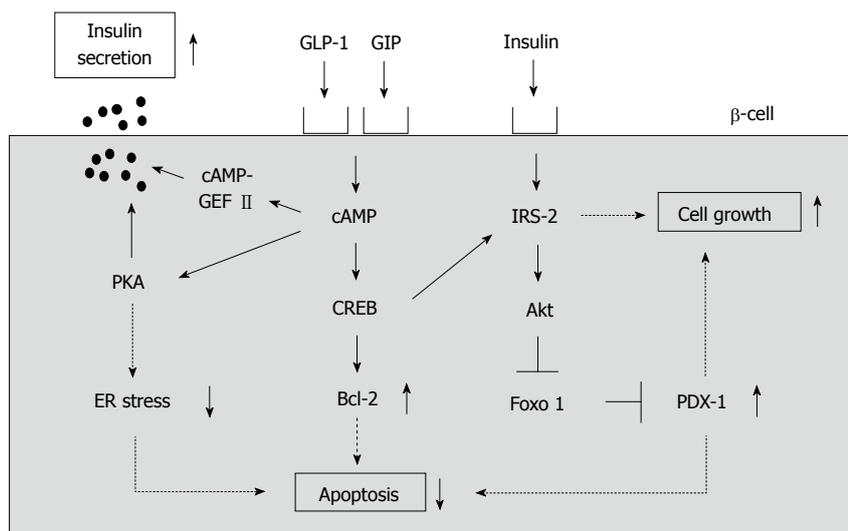
exposed to oxidative stress, both intrinsically expressed PDX-1 and exogenously introduced PDX-1 moved from the nuclei to the cytoplasm<sup>[49]</sup>. DN-JNK overexpression inhibited the oxidative stress-induced PDX-1 translocation, suggesting that activation of the JNK pathway is involved in PDX-1 translocation by oxidative stress. Furthermore, leptomycin B, a specific inhibitor of the classical, leucine-rich nuclear export signal (NES), inhibited nucleo-cytoplasmic translocation of PDX-1 induced by oxidative stress<sup>[49]</sup>. Taken together, it is likely that oxidative stress induces nucleo-cytoplasmic translocation of PDX-1 through activation of the JNK pathway, which leads to reduction of its DNA binding activity and suppression of insulin biosynthesis (Figure 2).

The forkhead transcription factor Foxo1 is known as one of the important fundamental transcription factors playing a key role in the process of apoptosis, cellular proliferation and differentiation, and glucose metabolism through regulating the transcription of various target genes<sup>[50,51]</sup>. Foxo1 regulates hepatic gluconeogenesis and thus contributes to insulin resistance<sup>[52]</sup>. Insulin inhibits the function of Foxo1 through Akt/PKB-mediated phosphorylation and nuclear exclusion<sup>[53]</sup>, and thereby suppresses hepatic gluconeogenesis. In addition, Foxo1 exhibits a counter localization to PDX-1 in  $\beta$ -cells<sup>[54]</sup>, suggesting that it is involved in the deterioration of  $\beta$ -cell function. Moreover, Foxo1 plays a role as a mediator between the JNK pathway and PDX-1<sup>[55]</sup>. In  $\beta$ -cell-derived cell line HIT cells, Foxo1 changed its intracellular localization from the cytoplasm to the nucleus after exposure to oxidative stress. In contrast to Foxo1, the nuclear expression of PDX-1 was decreased and its cytoplasmic distribution was increased by oxidative stress. Activation of the JNK pathway also induced the nuclear localization of Foxo1, whereas suppression of the JNK pathway reduced the oxidative stress-induced nuclear localization of Foxo1, suggesting an involvement of the JNK pathway in Foxo1 translocation<sup>[55]</sup>. In addition, oxidative stress or activation of the JNK pathway decreased Akt phosphorylation in HIT cells, leading to the decreased phosphorylation of Foxo1 following nuclear localization. Furthermore, adenoviral Foxo1 overexpression reduced the nuclear expression of PDX-1, whereas suppression of Foxo1 by

Foxo1-specific small interfering RNA retained the nuclear expression of PDX-1<sup>[55]</sup>. Taken together, oxidative stress and subsequent activation of the JNK pathway induce nuclear translocation of Foxo1 through the modification of the insulin signaling in  $\beta$ -cells, which leads to the nucleo-cytoplasmic translocation of PDX-1 and reduction of its DNA binding activity (Figure 2).

### MOLECULAR MECHANISM FOR DOWN-REGULATION OF NUCLEAR MAFA EXPRESSION UNDER DIABETIC CONDITIONS: POSSIBLE INVOLVEMENT OF OXIDATIVE STRESS AND SUBSEQUENT INDUCTION OF C-JUN EXPRESSION

It is known that c-Jun protein level and activity are increased in response to oxidative stress in various cells<sup>[56,57]</sup>. We recently reported that c-Jun expression was not clearly detected in islets of control m/m mice and young diabetic db/db mice, but that the number of c-Jun-positive cells gradually increased with age in the islets of diabetic db/db mice<sup>[58]</sup>. This expression pattern of c-Jun paralleled the loss of insulin gene transcription factor MafA expression. Quantitative real-time PCR analysis using freshly isolated islets from db/db mice clearly showed that c-Jun mRNA level was significantly increased but that both MafA and insulin mRNA levels were markedly decreased with age<sup>[58]</sup>. These results imply that the increased level of c-Jun caused a decrease in MafA and insulin gene expression in old diabetic mice. Furthermore, in immunostaining, in db/db mice nuclear MafA expression in pancreatic islets was markedly decreased with age and was not clearly detected in old mice, whereas in control m/m mice MafA expression retained up to old age<sup>[58]</sup>. In db/db mice insulin expression was also decreased in some  $\beta$ -cells in which MafA was undetectable or weakly expressed. Furthermore, MafA and insulin expression was suppressed in most c-Jun-positive  $\beta$ -cells. Similarly, in islets of diabetic KKAy mice, the number of c-Jun-positive  $\beta$ -cells was increased with marked hyperglycemia, and both MafA



**Figure 3** A role of incretin signaling in pancreatic  $\beta$ -cells. Incretins (GLP-1 and GIP) bind their incretin receptors (GLP-1 and GIP receptors) in  $\beta$ -cells and increase intracellular cAMP levels, leading to stimulation of insulin secretion, suppression of  $\beta$ -cell apoptosis and increase of  $\beta$ -cell growth.

and insulin protein levels were decreased in those cells<sup>[58]</sup>. These findings suggest that c-Jun is involved in the suppression of MafA and insulin expression under diabetic conditions. In addition, c-Jun overexpression markedly decreased insulin promoter activity, which was consistent with previous reports<sup>[59,60]</sup> (Figure 2).

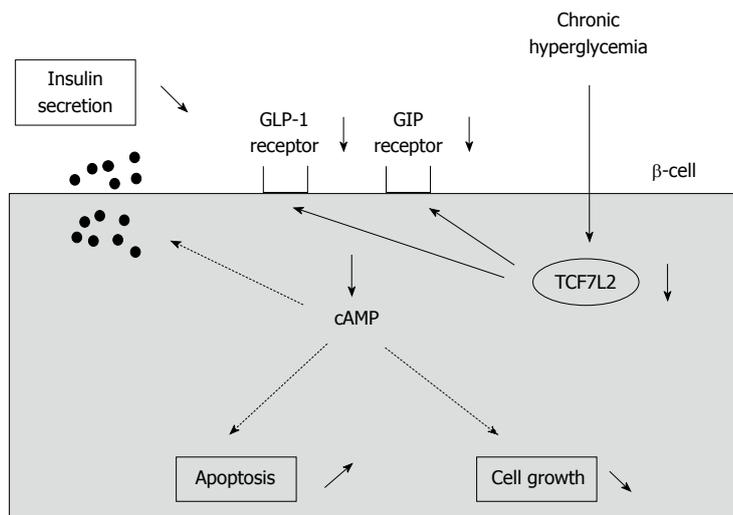
Although c-Jun protein expression was almost undetectable in MIN6 cells, adenoviral c-Jun overexpression markedly suppressed MafA protein level and its DNA-binding activity in MIN6 cells<sup>[58]</sup>. Northern blotting and real-time PCR analysis also showed that c-Jun overexpression significantly suppressed MafA mRNA level. Adenoviral overexpression of c-Jun in isolated mouse islets also markedly suppressed MafA mRNA and protein levels. Consistent with these results, mRNA levels of insulin 1 and 2 and insulin content were suppressed by c-Jun overexpression in both MIN6 cells and islets<sup>[58]</sup>. These findings directly demonstrate that c-Jun suppresses the expression of both MafA and insulin. In addition, since MafA appears to not only regulate insulin expression but also to be involved in insulin secretion<sup>[61,62]</sup>, it is likely that the suppression of MafA protein levels by c-Jun leads to insulin secretory defects that are often observed under diabetic conditions. In conclusion, the augmented expression of c-Jun in diabetic islets decreases MafA activity followed by reduced insulin biosynthesis and secretion, and thereby explains, at least in part, the molecular mechanism for  $\beta$ -cell glucose toxicity that is often observed in type 2 diabetes (Figure 2).

### MOLECULAR MECHANISM FOR DOWN-REGULATION OF INCRETIN RECEPTOR EXPRESSION IN $\beta$ -CELLS UNDER DIABETIC CONDITIONS: POSSIBLE INVOLVEMENT OF HYPERGLYCEMIA AND SUBSEQUENT REDUCTION OF TCF7L2 EXPRESSION

The incretin effect causes more insulin to be secreted

when glucose is orally taken compared to when given intravenously, even when blood glucose levels have the same profile. This effect is thought to be very important for maximizing insulin response during meals, thereby limiting postprandial glucose excursions. Two incretins have been identified: glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). It is thought that such incretins play an important role in glucose homeostasis by promoting insulin secretion immediately on meal ingestion. It is well known that incretins (GLP-1 and GIP) bind their incretin receptors (GLP-1 and GIP receptors) in  $\beta$ -cells and increase intracellular cAMP levels, leading to stimulation of insulin secretion, suppression of  $\beta$ -cell apoptosis and increase of  $\beta$ -cell growth (Figure 3).

Although plasma GLP-1 and GIP levels after meals are almost normal in type 2 diabetes, striking abnormalities are observed in the action of incretin hormones in type 2 diabetes<sup>[63]</sup>. It was reported that GLP-1 and GIP receptor expression was decreased in a glucose-dependent manner in islets isolated from 90% pancreatectomized diabetic (Px) rats<sup>[64]</sup>. Such decrease was not observed after normalization of blood glucose levels with phlorizin which is known to lower blood glucose levels by preventing glucose reabsorption from the glomerular filtrate in the kidney. These results suggest that hyperglycemia *per se* leads to down-regulation of GLP-1 and GIP receptor expression. Furthermore, insulin response to GLP-1 or GIP was markedly reduced in islets isolated from diabetic rats compared to those from control rats<sup>[65]</sup>. These results indicate that down-regulation of GLP-1 and GIP receptor expression leads to the deterioration of  $\beta$ -cell function. Similar results were reported in obese type 2 diabetic db/db mice; incretin receptor expression in islets was markedly decreased at 16 wk of age in db/db mice but was preserved by normalization of blood glucose levels with insulin therapy<sup>[66]</sup>. These results strengthen the hypothesis that hyperglycemia *per se* leads to down-regulation of GLP-1 and GIP receptor expression (Figure 4).



**Figure 4 Down-regulation of incretin receptors in pancreatic  $\beta$ -cells under diabetic conditions.** Under diabetic conditions, expression of incretin receptors (GLP-1 and GIP receptors) in  $\beta$ -cells are down-regulated, leading to decrease of insulin secretion, increase of  $\beta$ -cell apoptosis and decrease of  $\beta$ -cell growth.

Furthermore, down-regulation of GLP-1 and GIP receptor expression was observed in type 2 diabetic subjects, as observed in diabetic rodents<sup>[66]</sup>. In addition, it was shown that transcription factor TCF7L2 is involved in down-regulation of GLP-1 and GIP receptor expression found under diabetic conditions<sup>[66]</sup>. Recent human genetics studies have revealed that common variants of the *TCF7L2* gene are strongly associated with type 2 diabetes mellitus. It was shown that expression levels of GLP-1 and GIP receptors were lower in islets of type 2 diabetic subjects as well as in isolated human islets treated with siRNA to TCF7L2 (siTCF7L2). Insulin secretion stimulated by glucose, GLP-1 or GIP was also impaired in siTCF7L2-treated isolated human islets. In conclusion, we think that the down-regulation of incretin receptors by hyperglycemia is largely responsible for the impaired incretin effects and thus, at least in part, explains the molecular mechanism for  $\beta$ -cell dysfunction found in diabetes (Figure 4).

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WJD 5<sup>th</sup> Anniversary Special Issues (2): Type 2 diabetes

## Type 2 diabetes mellitus in children and adolescents

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## Abstract

Type 2 diabetes mellitus is emerging as a new clinical problem within pediatric practice. Recent reports indicate an increasing prevalence of type 2 diabetes mellitus in children and adolescents around the world in all ethnicities, even if the prevalence of obesity is not increasing any more. The majority of young people diagnosed with type 2 diabetes mellitus was found in specific ethnic subgroups such as African-American, Hispanic, Asian/Pacific Islanders and American Indians. Clinicians should be aware of the frequent mild or asymptomatic manifestation of type 2 diabetes mellitus in childhood. Therefore, a screening seems meaningful especially in high risk groups such as children and adolescents with obesity, relatives with type 2 diabetes mellitus, and clinical features of insulin resistance (hypertension, dyslipidemia, polycystic ovarian syndrome, or acanthosis nigricans). Treatment of choice is lifestyle intervention followed by pharmacological treatment (e.g., metformin). New drugs such as dipeptidyl peptidase inhibitors or glucagon like peptide 1 mimetics are in the pipeline for treatment of youth with type 2 diabetes mellitus. However, recent reports indicate a high dropout of the medical care system of adolescents with type 2 diabetes mellitus suggesting that management of children and adolescents with type 2 diabetes mellitus requires some remodeling of cur-

rent healthcare practices.

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**Key words:** Type 2 diabetes mellitus; Children; Clinical manifestation; Screening; Comorbidity; Treatment**Core tip:** Review of the current stage of diagnosis and treatment of type 2 diabetes in children and adolescents.

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## INTRODUCTION

Thirty years ago, type 2 diabetes mellitus has been thought to be a rare occurrence in children and adolescents. However, in the mid-1990s, investigators began to observe an increasing incidence of type 2 diabetes mellitus worldwide<sup>[1]</sup>. This is particularly the case in the United States<sup>[1,2]</sup> but has also been reported in other countries like Canada, Japan, Austria, United Kingdom and Germany<sup>[3-10]</sup>. In some regions in the United States, type 2 diabetes mellitus is as frequent as type 1 diabetes mellitus in adolescents<sup>[11]</sup>.

This observation followed a striking increase both the prevalence and the degree of obesity in children and adolescents in many populations<sup>[12,13]</sup>. Overweight is at present the most common health problem facing children in both develop and developing countries<sup>[13]</sup>. While obesity is not increasing any more in the United States and some countries in Europe<sup>[14,15]</sup>, the prevalence of type 2 diabetes mellitus has been increased threefold<sup>[15]</sup>. This has been attributed to the fact, that the prevalence of obesity is not increasing but the degree of obesity in affected children and adolescents<sup>[15]</sup>.

Type 2 diabetes mellitus is a serious and costly dis-

ease. The chronic complications of diabetes mellitus include accelerated development of cardiovascular diseases, end-stage renal disease, loss of visual acuity, and limb amputations. All of these complications contribute to the excess morbidity and mortality in individuals with diabetes mellitus.

Since the incidence and prevalence of type 2 diabetes mellitus in children are increasing and if this increase cannot be reversed, our society will face major challenges. That is, the burden of diabetes mellitus and its complications will affect many more individuals than currently anticipated, and the cost of diabetes mellitus to our society will cause us to consume enormous resources.

## PATHOPHYSIOLOGY OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

Type 2 diabetes mellitus is a complex metabolic disorder of heterogeneous etiology with social, behavioral, and environmental risk factors unmasking the effects of genetic susceptibility<sup>[16]</sup>. There is a strong hereditary (likely multigenic) component to the disease, with the role of genetic determinants illustrated when differences in the prevalence of type 2 diabetes mellitus in various racial groups are considered<sup>[17]</sup>. Although substantial progress in our knowledge of the genetic basis of type 2 diabetes mellitus is taking place, these new discoveries represent but a small proportion of the genetic variation underlying the susceptibility to this disorder<sup>[17]</sup>. Furthermore, the recent increases observed in diabetes mellitus prevalence are too quickly to be the result of increased gene frequency and altered gene pool, emphasizing the importance of environmental factors.

Glucose homeostasis depends on the balance between insulin secretion by the pancreatic  $\beta$ -cells and insulin action. It is well recognized that insulin resistance to insulin-stimulated glucose uptake is a characteristic finding in patients with type 2 diabetes mellitus and impaired glucose metabolism. The evolution from normal to impaired glucose tolerance (IGT) is associated with a worsening of insulin resistance. Impaired glucose tolerance is an intermediate stage in the natural history of type 2 diabetes mellitus and is a predictor of the risk of developing diabetes mellitus and cardiovascular disease<sup>[7,10,11,18]</sup>. However, there is a high spontaneous conversion rate from IGT to normal glucose tolerance in the next 3 to 5 years in children and adolescents with impaired glucose tolerance<sup>[19,20]</sup>. This normalization has been attributed to changes of insulin resistance at end of puberty.

Puberty appears to play a major role in the development of type 2 diabetes mellitus in children<sup>[11]</sup>. During puberty, there is increased resistance to the action of insulin, resulting in hyperinsulinemia. After puberty, basal and stimulated insulin responses decline. Hyperinsulinemic- euglycemic clamp studies demonstrated

that insulin-mediated glucose disposal is on average 30% lower in adolescents between Tanner stage II and IV compared with prepubertal children and with young adults. Increased growth hormone secretion in puberty is discussed to be responsible for the insulin resistance during puberty<sup>[21]</sup>. Given this information, it is not surprising that the peak age at presentation of type 2 diabetes mellitus in children coincides with the usual age of mid-puberty<sup>[3,11]</sup>.

For diabetes mellitus to develop insulin resistance alone is not sufficient and inadequate  $\beta$ -cell insulin secretion is necessary<sup>[7,10,11,18]</sup>. In patients with type 2 diabetes mellitus, impaired insulin action and insulin secretory failure are both present. It has been proposed that hyperglycemia may worsen both insulin resistance and insulin secretory abnormalities, thus enhancing the transition from impaired glucose tolerance to diabetes mellitus<sup>[11]</sup>.

The adverse effect of obesity on glucose metabolism is evident early in childhood. Obese children are hyperinsulinemic and have approximately 40% lower insulin-stimulated glucose metabolism compared with non-obese children<sup>[22]</sup>. Furthermore, the inverse relationship between insulin sensitivity and abdominal fat is stronger for visceral than for subcutaneous fat<sup>[22,23]</sup>.

It is interesting to note that adipose tissue expanding in the obese state synthesizes and secretes metabolites and signaling proteins like leptin, adiponectin, and tumor necrosis factor- $\alpha$ . These factors are known to alter insulin secretion and sensitivity and even cause insulin resistance under experimental and clinical conditions<sup>[24]</sup>.

Racial differences in insulin sensitivity are also evident in childhood. African-American 7- to 11-year-old children have significantly higher insulin levels than age-matched white children<sup>[25]</sup>. These data suggest that particular ethnic groups may have a genetic predisposition to insulin resistance, which may increase their risk for type 2 diabetes mellitus. In concordance, obese Swedish children have higher fasting glucose levels than obese German children<sup>[26]</sup>.

## EPIDEMIOLOGY OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

The prevalence of type 2 diabetes mellitus in children and adolescents in the United States is approximately 12:100000<sup>[15,27,28]</sup>, while it is still rare in Europe (approximately 2.5:100000)<sup>[8,29]</sup>. The majority of young people diagnosed with type 2 diabetes mellitus was found in specific ethnic subgroups such as African-American, Hispanic, Asian/Pacific Islanders and American Indians being highest in Pima Indians (22.3/1000 in 10 - 14-year-old children)<sup>[30]</sup>. Furthermore, the great majority of the children were obese. Screening studies in obese adolescents have reported a prevalence of 0.4% up to 1% of type 2 diabetes mellitus in obese children  $\geq 12$  years<sup>[3,31,32]</sup>. Within the whole pediatric cohort surveyed,

**Table 1 Comparison between Caucasian and non-Caucasian children and adolescents with type 2 diabetes mellitus**

	Caucasian	Non-Caucasian
Mean age at onset in years	14	12
Gender	Female > male	Female > male
Clinical manifestation	50% asymptomatic 4% ketoacidosis	33% asymptomatic 5%-25% ketoacidosis
Obese	90%	90%
Acanthosis nigricans	50%	90%
1 <sup>st</sup> - and 2 <sup>nd</sup> - degree relatives with type 2 diabetes mellitus	83%	74%-100%

Table adapted from Reference [3].

the overall incidence of type 2 diabetes mellitus remained low when compared with type 1 diabetes mellitus. This had led some researchers to question the claims of an “epidemic” of pediatric type 2 diabetes mellitus<sup>[33]</sup>, although there is general agreement that type 2 diabetes mellitus in youth appears to be emerging as a serious clinical issue<sup>[9,34]</sup>.

## CLINICAL PRESENTATION OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

Obesity is the hallmark of type 2 diabetes mellitus<sup>[10]</sup>. Most children with type 2 diabetes mellitus are obese or extremely obese at diagnosis and present with glucosuria without ketonuria, absent or mild polyuria and polydipsia, and little or no weight loss<sup>[3,10]</sup>. Currently, children with type 2 diabetes mellitus are usually diagnosed over the age of 10 years and are in middle to late puberty<sup>[3,10,11]</sup>.

In the type 2 diabetes mellitus mildest form, the diagnosis is made in an asymptomatic child during a routine medical check-up by detection of hyperglycaemia or glycosuria<sup>[10]</sup>. One third of patients are diagnosed by urinalysis during routine physical examination<sup>[10,11]</sup>. In its severest form, the child presents with polyuria, polydipsia, and weight loss. Up to 33% in particular ethnic groups have ketonuria at diagnosis and 5%-25% ketoacidosis at presentation<sup>[10,11]</sup> (Table 1). Vary rare, type 2 diabetes mellitus manifest with a hyperglycaemic hyperosmolare coma. With these clinical pictures, often the distinction from type 1 diabetes mellitus is not possible until months later, when insulin requirements decline and a non-insulin-dependent course develops without dependence on insulin for survival.

Children with type 2 diabetes mellitus frequently have a family history of type 2 diabetes mellitus and those of non-European ancestry (Americans of African, Hispanic, Asian, and American Indian descent) are disproportionately represented. Of the patients, 74%-100% have a first- or second-degree relative with type 2 diabetes mellitus<sup>[10]</sup>. Of note, diabetes mellitus in parents or other relatives may not be recognized until the child is diagnosed.

Acanthosis nigricans and polycystic ovarian syndrome (PCOS), disorders associated with insulin resistance and obesity, are common in youth with type 2 diabetes mellitus<sup>[10]</sup>. Acanthosis is a cutaneous finding characterised by velvety hyperpigmented patches most prominent in intertriginous area. It is present in up to 50 up to 90% of children with type 2 diabetes mellitus. It is recognised more frequently in darker-skinned obese individuals. PCOS is characterised by hyperandrogenism and chronic anovulation. Lipid disorders and hypertension also occur more frequently in children with type 2 diabetes mellitus.

Most of the Caucasian children and adolescents with type 2 diabetes mellitus were asymptotically at diagnosis in contrast to other ethnicities (Table 1). The minority populations demonstrated at manifestation of diabetes mellitus frequently more symptoms and higher insulin and C-peptide levels<sup>[11]</sup>.

Some syndromes such as Klinefelter syndrome, Bardet Biedl Syndrome, Prader Willi Syndrome and Alström Syndrom are associated with type 2 diabetes mellitus. They all are associated with mental retardation and frequently to extreme obesity.

## DIFFERENTIAL DIAGNOSIS OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

Individuals with type 2 diabetes mellitus may have clinical presentations indistinguishable from those of patients with other types of diabetes mellitus<sup>[10]</sup>. This is relevant because as the number of children with type 2 diabetes mellitus increases, it becomes increasingly important to classify their diabetes mellitus correctly so that appropriate therapy may be instituted.

Typically, children with type 1 diabetes mellitus are not overweight and have recent weight loss, polydipsia, and polyuria (Table 2)<sup>[10]</sup>. They have a short duration of symptoms and frequently have ketoacidosis at presentation<sup>[7,10,35]</sup>. After metabolic stabilization, they may have an initial period of diminished insulin requirement, after which they require insulin for survival.

It should be highlighted, that none of symptoms and factors in Table 2 relate to a definite diagnosis. For example, the increased prevalence of type 2 diabetes mellitus within the whole population means that many individuals with type 1 diabetes mellitus are now more likely to have a family history of type 2 diabetes mellitus. Likewise, type 1 diabetes mellitus also presents in overweight/obese individuals and indeed the prevalence of weight-related problems in type 1 diabetes mellitus appears to be significant<sup>[36]</sup>.

Even if type 2 diabetes mellitus is defined by the absence of  $\beta$ -cell autoantibodies<sup>[7,18]</sup>, some studies reported of nearly 30%  $\beta$ -cell autoantibodies in European children and adolescents clinically appearing as type 2 diabetes mellitus<sup>[4,11,37]</sup>. These children have a non-insulin dependent children over a period > 1 year. The possibility that

**Table 2 Clinical characteristics of type 1, type 2 and MODY diabetes mellitus**

Clinical characteristic	Type 1 diabetes mellitus	Type 2 diabetes mellitus	MODY diabetes mellitus
Age when diagnosis is established	Preschool-adolescents	> 10 yr	MODY 2: youth MODY 3: adolescents
Obesity	Uncommon <sup>1</sup>	Common	Uncommon
Gender	Male = female	Female > male	Male = female
Relatives	5% Type 1 D.m.	75%-100% Type 2 D.m.	100% MODY
Population	Predominantly Caucasian	Predominantly Americans of African, Hispanic, Asian, and American Indian	
b-cell autoantibodies	85%-98%	Uncommon <sup>2</sup>	Uncommon
Insulin, C-peptide	Low	high	Low
Ketoacidosis	Frequently	< 33%	Uncommon
Associated disorders	Autoimmune disorders (thyroid, adrenal, vitiligo), celiac disease	Acanthosis nigricans PCOS Metabolic Syndrome	MODY 5: urogenital malformation MODY 8: exocrine pancreas insufficiency

<sup>1</sup>But prevalence of overweight children with type 1 diabetes mellitus is increasing parallel to the obesity epidemic; <sup>2</sup>Children with type 2 diabetes mellitus and b-cell autoantibodies have been reported. MODY: Maturity onset of diabetes mellitus in youth.

positive  $\beta$ -cell antibodies in these non insulin-requiring diabetic children and adolescents represent a form of early-onset latent autoimmune diabetes mellitus similar to that described in adults (LADA: latent autoimmune diabetes mellitus of the adult) needs to be considered. Worldwide studies have identified 10%-20% of diabetic patients with  $\beta$ -cell autoantibodies in non-insulin-requiring adult diabetics<sup>[38-40]</sup>. Patients with LADA share insulin resistance with type 2 diabetes mellitus patients but display a more severe defect in  $\beta$ -cell capacity<sup>[41]</sup>. Following the terminology "latent autoimmune diabetes mellitus in adulthood", the non-insulin dependent diabetic children and adolescents with  $\beta$ -cell autoantibodies could be named "latent autoimmune diabetes mellitus in youth" (LADY). Double diabetes mellitus or type 1.5 diabetes mellitus are other proposed names for this entity.

The accelerator hypothesis postulates a shared basis for both type 1 and type 2 diabetes mellitus: Besides individual predisposition and autoimmunity, insulin resistance is discussed to lead to  $\beta$ -cell insufficiency<sup>[42,43]</sup>. In the prediabetic period of an immune-mediated destruction of  $\beta$ -cells, increasing insulin resistance can result in clinical diabetes mellitus<sup>[38]</sup>. Obesity and puberty are important factors for developing insulin resistance in childhood and adolescence<sup>[22]</sup>. Apart from  $\beta$ -cell autoantibodies in children clinically classified as type 2 diabetes mellitus, negative autoantibodies in children with type 1 diabetes mellitus with acute-onset of diabetes mellitus, severe metabolic impairment and insulin requirement only in the early stage of disease are reported<sup>[7,9]</sup>. In conclusion, type 1 and type 2 diabetes mellitus do not seem to be completely distinctive and can overlap considerably. Therefore, serology cannot completely distinguish these two types of diabetes mellitus.

Maturity-onset of diabetes mellitus of the young (MODY) is another rare form of diabetes mellitus in children that includes several disorders caused by monogenic defects in  $\beta$ -cell function<sup>[44]</sup>. MODY 2 (defect in glucokinase) and MODY 3 (defect in HNF1 $\alpha$ ) are the most frequent types of MODY. Patients with MODY have a dominant genetic trait, usually are non-obese and

have low fasting insulin levels. Recent studies suggest that the clinical presentation of MODY is broad, ranging from asymptomatic hyperglycemia to a severe acute presentation. MODY has been reported in all races/ethnicities. These gene abnormalities are thought to be rare, and molecular diagnostic testing.

## DIAGNOSTIC CRITERIA OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

The criteria for diagnosis of diabetes mellitus in children and adolescents are symptoms of diabetes mellitus such as polydipsia, polyuria, and unexplained weight loss plus casual glucose concentration  $\geq 200$  mg/dL (11.1 mmol/L) in venous plasma, fasting glucose  $\geq 126$  mg/dL (7.0 mmol/L) in venous or capillary plasma, or two-hours glucose during oGTT  $\geq 200$  mg/dL (11.1 mmol/L) in venous plasma or capillary whole blood sample. Recently revised American Diabetes mellitus Association (ADA) criteria allow utilization of hemoglobin A1c (HbA1c)  $\geq 6.5\%$  for diagnosis of diabetes mellitus<sup>[18,45]</sup>. In case of asymptomatic manifestation, fasting glucose, HbA1c, or oGTT test has to be repeated on another day for diagnosis<sup>[7,10,18]</sup>.

In most patients with diabetes mellitus, classification can be made reliably on the basis of clinical presentation and course<sup>[7,10,18]</sup>. In the unusual circumstance that requires a specific classification to be made, other test may be necessary, such as fasting insulin or C-peptide determination and occasionally,  $\beta$ -cell autoantibodies measurements (Figure 1). To achieve a high degree of sensitivity, a combination of test is required, which greatly increases the cost of classification.

C-peptide levels are elevated in individuals with type 2 diabetes mellitus in contrast to patients with type 1 diabetes mellitus or MODY diabetes. Specific autoantibodies to insulin, to GAD- $\text{II}$ , or to tyrosine phosphatases insulin antibodies (IA)-2 and IA-2b are found at presentation in 85%-98% of individuals with immune-mediated type 1 diabetes mellitus<sup>[7,35]</sup>. Type 1 diabetes

Table 3 Criteria for testing of type 2 diabetes mellitus in children and adolescents
Overweight (BMI > 90 percentile) plus one of the following risk factors:
Family history of type 2 diabetes mellitus in first- or second-degree relative
Race/ethnicity (Asian, American Indian, Africa-Americans, Hispanics)
Signs of insulin resistance or conditions associated with insulin resistance (acanthosis nigricans, hypertension, dyslipidemia, polycystic ovarian syndrome)
Extreme obesity (BMI > 99.5 percentile)

Table adapted from Reference [7]. BMI: Body mass index.

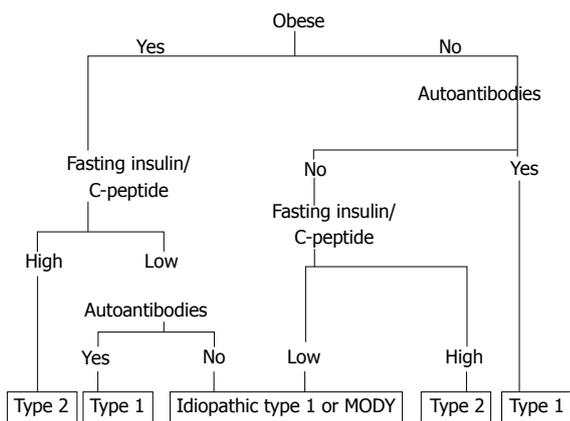


Figure 1 Flow sheet for classification of diabetes mellitus in children and adolescents. Adapted from Reference [7], cave autoantibodies also appear in type 2 diabetes mellitus. MODY: Maturity onset of diabetes mellitus in youth.

mellitus also has a strong HLA association; however, HLA typing is not a useful diagnostic tool.

## SCREENING FOR TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

Most of the European Caucasian children and adolescents with type 2 diabetes mellitus and one third of the American children were asymptotically at diagnosis. According to this, the prevalence in screening studies in Europe of obese children was much higher than the prevalence rate reported in the standardised documentation system of diabetes mellitus for example in Germany<sup>[32,46]</sup>. Therefore it is likely that, as with adults, undiagnosed type 2 diabetes mellitus is a common condition in childhood<sup>[47]</sup>. In conclusion, a screening of type 2 diabetes mellitus seems necessary since unrecognised hyperglycaemia would undoubtedly contribute to both microvascular and macrovascular risk in later life<sup>[11]</sup>.

However, at the present time, a general screening for type 2 diabetes mellitus in youth is unlikely to be cost-effective. Therefore a targeted screening seems to be necessary. Consistent with the recommendations for screening in adults, only children at substantial risk for the presence or the development of type 2 diabetes mellitus should be tested. Screening in high-risk population demonstrated type 2 diabetes mellitus in approximately

1% of obese Caucasian children in Germany<sup>[32,46]</sup> and in 4% of screened obese adolescents in particular ethnic groups in the United States<sup>[31]</sup>. The ADA recommends a screening in overweight children and adolescents at onset of puberty in high risk patients (Table 3)<sup>[7]</sup>. Testing should be performed every 2 years starting at the age of 10 years or at onset of puberty if it occurs in a younger age by fasting glucose or oGTT<sup>[7]</sup>.

However, these screening recommendations have some pitfalls. Requirements for testing an asymptomatic group include the availability of a test that is sensitive (few false negatives) and accurate with acceptable specificity (minimal number of false positive). Since fasting blood glucose failed to diagnose diabetes mellitus in one fourth of children with type 2 diabetes mellitus in the European cohort<sup>[3]</sup>, oGTT seems to be a better screening tool even if fasting glucose is preferred because of its lower costs and greater convenience. However, the low reproducibility of an oGTT has to be kept in mind<sup>[48]</sup>. Using the criteria (1) type 2 diabetes mellitus in the first or second degree relatives, (2) onset of puberty, and (3) extreme obesity allows to identify the great majority of overweight European children, which should be screened by oGTT, when two of this three criteria are fulfilled<sup>[49]</sup>. Studies using HbA1c as screening tool were disappointing, since one third of the asymptomatic children with type 2 diabetes mellitus demonstrated normal values<sup>[3]</sup>. The standardisation process of the HbA1c may have improved its screening value, since HbA1c has been included in the ADA recommendation for the diagnosis of diabetes mellitus<sup>[18,45]</sup>. However, studies testing the usefulness of HbA1c after the standardisation process as screening tool are missing yet.

## COMPLICATIONS OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

The chronic complications of diabetes mellitus in adults include macrovascular disease like accelerated development of cardiovascular disease leading to stroke and myocardial infarction, and microvascular diseases like retinopathy, nephropathy and neuropathy leading to end-stage renal disease, loss of visual acuity, and limb amputations. All of these complications contribute to the excess morbidity and mortality in individuals with diabetes mellitus. One notable outcome of the UK Prospective Diabetes mellitus Study (UKPDS) analysis was the observation that the accrual of endpoints was a time dependent process<sup>[50]</sup>. Therefore, children and adolescents with type 2 diabetes mellitus have a higher risk for complication as compared to adults with diabetes mellitus. Accordingly, developing type 2 diabetes mellitus at a younger age is also associated with a much higher risk of long-term cardiovascular disease than those who develop type 2 diabetes mellitus in middle age<sup>[51]</sup>. Young people with type 2 diabetes mellitus appear to be at a much higher risk of developing early diabetes mellitus

associated complications than those with type 1 diabetes mellitus. This higher level of risk does not appear to be related to overall levels of glycemic control or duration of disease but to occurrence of hypertension and dyslipidemia<sup>[52]</sup>. These cardiovascular risk factors are more frequent in adolescents suffering from type 2 diabetes mellitus compared to type 1 diabetes mellitus<sup>[35]</sup>. In the TODAY study, 14% of adolescents with type 2 diabetes mellitus suffered from hypertension, 80% demonstrated low HDL concentrations, and 10% had hypertriglyceridemia<sup>[35]</sup>. In the SEARCH study, 92% of the adolescents with type 2 diabetes mellitus fulfilled the definition of metabolic syndrome<sup>[2]</sup>. These prevalences are similar to cardiovascular risk factors in European adolescents with type 2 diabetes mellitus<sup>[4,53]</sup>.

We know little about the onset and progress of macrovascular disease in children and adolescents with type 2 diabetes mellitus. Arteriosclerosis is a time dependent phenomenon, and thus the absolute time from diagnosis to developing pathological cardiovascular lesions may be many years - in that sense these children may be protected by age since they do not have pre-existing age related cardiovascular disease. However, already adolescents with type 2 diabetes mellitus demonstrated an increased intima-media thickness<sup>[54]</sup>, which is predictive for heart attack and stroke<sup>[55]</sup>.

Microvascular disease is the hallmark of hyperglycaemia diagnosed at a young age. Data from Japanese, Pima Indian children show the presence of microvascular diabetic complications already at diagnosis and follow-up<sup>[1]</sup>. In Japanese children, incipient retinopathy was detected in 36% of the cases at the time of diagnosis, and in 39% of the cases at 2 years follow-up, while microalbuminuria was observed in 39% at 2 years follow-up<sup>[56]</sup>. Among Pima Indian children, 22% had microalbuminuria, and at follow-up between 20 and 29 years of age 60% had microalbuminuria and 17% had already macroalbuminuria<sup>[57]</sup>. In the SEARCH study, 4% of the adolescents with type 2 diabetes mellitus demonstrated retinopathy and 28% microalbuminuria<sup>[2]</sup>. In contrast, in European adolescents with type 2 diabetes mellitus no retinopathy and only 5% microalbuminuria was reported<sup>[4,53]</sup> suggesting genetic differences.

## TREATMENT OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

The American Academy of Pediatrics has, very recently, published management guidelines how to treat children and adolescents with type 2 diabetes mellitus<sup>[9]</sup>. The ideal goal of treatment is normalization of blood glucose values and HbA1c<sup>[10]</sup>. Successful control of the associated comorbidities, such as hypertension and dyslipidemia, is also important<sup>[10]</sup>. The ultimate goal of treatment is to decrease the risk of acute and chronic complications associated with diabetes mellitus.

Most of the recommended guidelines for treatment in children with type 2 diabetes mellitus are extrapolated from experience gained in adults<sup>[7,9,10]</sup>. Despite of severe manifestation, initial management of obese children and

adolescents with type 2 diabetes mellitus should consist of behaviour modification strategies for lifestyle change such as decreasing high-caloric high-fat food choice and sedentary behaviour, while increasing physical activity<sup>[58]</sup>. Weight control is essential for reaching treatment goals and are effective to treat type 2 diabetes mellitus in adolescents<sup>[59]</sup>. However, lifestyle changes cannot be imposed and self-motivation is necessary<sup>[60,61]</sup>. Referral to a dietician with knowledge and experience in nutritional management of children with diabetes mellitus is necessary<sup>[60]</sup>. Dietary recommendations should be culturally appropriate, sensitive to the family resources, and provided to all caregivers. Encouraging healthy eating habits by the entire family is important<sup>[58,60]</sup>.

All children with type 2 diabetes mellitus should receive comprehensive self-management education<sup>[1,60]</sup>: Self-management education should include teaching self-monitoring of blood glucose (SMBG). SMBG should be performed as needed and during periods of acute illness or when symptoms of hyper- or hypoglycaemia occur. Patients on insulin should also monitor periodically for asymptomatic hypoglycaemia. Routine blood glucose monitoring should be tailored to individual needs but should probably include a combination of fasting and postprandial glucose measurements. HbA1c should be assayed to monitor glycemic control.

Since only a few of youths with type 2 diabetes mellitus can be treated with diet and exercise alone<sup>[61]</sup>, pharmacological intervention is frequently required to achieve normoglycemic state.

## PHARMACOLOGICAL TREATMENT OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

If treatment goal (HbA1c < 7%) with nutrition education and exercise is not met, pharmacological therapy is indicated<sup>[9,10]</sup>. Many drugs are available for individuals with type 2 diabetes (Table 4), although only metformin and insulin are currently licensed for use for under 18-year-old<sup>[10]</sup>. Most paediatric diabetologists use oral agents for children with type 2 diabetes mellitus. Advantages of oral agents include potentially greater compliance and convenience for the patient. Clinical features suggesting initial treatment with insulin include dehydration, presence of ketosis, and acidosis.

Metformin, a biguanide, is undoubtedly the most appropriate starting point for pharmacological treatment in children with type 2 diabetes mellitus. The effectiveness has been proven for adolescents in a randomized controlled trial<sup>[62]</sup>. Metformin decreases hepatic glucose output and enhances primarily hepatic and also muscle insulin sensitivity without a direct effect on  $\beta$ -cell function. Metformin has the advantage of weight reduction, decrease in lipids without the risk of hypoglycaemia. Because of concerns about lactic acidosis metformin is contraindicated in patients with impaired renal function

**Table 4 Treatment option of type 2 diabetes mellitus in children and adolescents [Federal Drug Administration (United States), European Medicine Evaluation Agency (European)]**

Modality	Glycaemia reduction	Enhance insulin secretion	Insulin resistance lowering	FDA/EMA approved for children	Notes
Diet and exercise	Yes	No	Yes	Yes	First-line approach Efficacy depends on successful life-style change
Insulin	Yes	No	No	Yes	Weight gain discussed risk of hypoglycemia
Metformin	Yes	No	Yes	Yes	Good safety record minimal weight loss
Sulphonylureas	Yes	Yes	No	No	Good safety record in adults risk of hypoglycemia
Meglitinide analogues	Yes	Yes	No	No	Sparse data on their use
Thiazolidinediones	Yes	?	Yes	No	Weight gain lack of long-term data
Dipeptidyl peptidase inhibitors (DPP4)/ glucagon like peptide (GLP) 1 mimetics	Yes	No	No	No	Moderate weight loss Carcinoma of the pancreas discussed
Sodium-Glucose Cotransporter 2 Inhibitors	Yes	No	No	No	Lack of long-term data Minimal weight loss Urinary and genital tract infections Risk of hypoglycaemia Sparse data on their use Lack of long-term data
Acarbose	?	No	No	No	Side effects may be unacceptable
Orlistat	?	No	No	No	Side effects may be unacceptable
Surgical treatment of obesity	Yes	No	Yes	No	Some anecdotal evidence

?: Unknown; FDA: Federal Drug Administration; EMA: European Medicine Evaluation Agency.

and should be discontinued with the administration of radiocontrast material or hypocaloric diet. Metformin should not be used in patients with known hypoxicemic conditions, severe infection, hepatic disease, or alcohol abuse. The most common side effects of metformin are gastrointestinal disturbances. The dose of metformin should be increased up to 2 g in split doses, unless there are gastrointestinal side effects. Metformin has a good safety record, but should not be given if there is any doubt at all about the nature of diagnosis.

If monotherapy with metformin is not successful over a reasonable period of time (3-6 mo), several alternatives can be considered. Other drugs, which are not approved for children and adolescents, have been less frequently used in children. No oral agent should be used during pregnancy. The use of rosiglitazone has been studied in a randomized trial in adolescents compared to lifestyle intervention and metformin in the TODAY study<sup>[63]</sup>. Monotherapy with metformin was associated with durable glycemic control in approximately half of children and adolescents with type 2 diabetes mellitus. The addition of rosiglitazone, but not an intensive lifestyle intervention, was superior to metformin alone. However, rosiglitazone has been withdrawn from the market due to its side effects and is not available any more.

Insulin treatment will often be the only feasible way of controlling hyperglycaemia. There is no specific contraindication in children. Insulin regimes should be adopted that are carefully tailored to lifestyle (bedtime insulin alone, twice-a-day insulin or multidose insulin regimes).

In those extremely obese adolescents where lifestyle change and pharmacotherapy have been unsuccessful,

then bariatric surgery may need to be considered<sup>[64]</sup>.

## MONITORING AND TREATMENT OF COMPLICATIONS OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

Since microvascular complications of type 2 diabetes mellitus like retinopathy and nephropathy already occur in children, dilated eye examinations should be performed<sup>[1,60]</sup>. Screening for microalbuminuria should also be performed yearly<sup>[1,7]</sup>. Angiotensin converting enzyme (ACE) inhibitors are the agents of choice in children with microalbuminuria<sup>[1,7]</sup>. It is unclear whether foot examinations are important in children<sup>[1,7]</sup>. Control of hypertension in children with type 2 diabetes mellitus is mandatory<sup>[1,7]</sup>. If normotension is not achieved by ACE inhibitors, combination therapy with  $\alpha$ -blockers, calcium antagonists or low-dose diuretics may be needed. Testing for and treating lipid abnormalities are necessary to avoid macrovascular diseases<sup>[1,7]</sup>.

## CHILDREN AND ADOLESCENTS WITH DIABETES MELLITUS TYPE 2: AN UNDERSERVED POPULATION

So far, our medical care system seems to be inadequate to care for children and adolescent with type 2 diabetes mellitus for several reasons<sup>[53]</sup>: (1) The majority of children and adolescents with type 2 diabetes mellitus remain undiagnosed: Screening studies in obese Cau-

casian children have reported a prevalence of 0.5% up to 1% of type 2 diabetes mellitus in obese children  $\geq 12$  years<sup>[5]</sup>. Based on the prevalence of obesity of 6.5% in Central Europe<sup>[12,65]</sup>, approximately 6000 children in Germany, Austria, and Switzerland suffer from type 2 diabetes mellitus. However, in standardized prospective documentation systems of children and adolescents in Central Europe by specialized pediatric diabetes mellitus and obesity treatment centers, only nearly every sixth ( $n = 1071$ ) child with type 2 diabetes mellitus has been registered<sup>[53]</sup>. This high prevalence of undiagnosed type 2 diabetes mellitus has also been reported in other countries<sup>[47]</sup>; (2) Children and adolescents with diagnosis of type 2 diabetes mellitus frequently get lost to follow-up early after diagnosis: In an European registry, 173 (80%) of 217 children and adolescents with type 2 diabetes mellitus were lost to follow-up after 4 mo, and only 18 children (8%) were transferred to specialized diabetes centers<sup>[53]</sup>. In another Central European database (the diabetes mellitus documentation system dpv), 620 (63%) of 991 children with type 2 diabetes mellitus were lost to follow-up after 4 mo<sup>[53]</sup>. These high frequencies of drop-out greater than 50% have also been reported in other ethnicities with type 2 diabetes mellitus in childhood<sup>[66,67]</sup>; and (3) The recommended therapy of lifestyle intervention to achieve weight loss in type 2 diabetes mellitus is not suitable for most patients: Lifestyle intervention led to a documented reduction of overweight in only 17% of the children treated in pediatric diabetes centers<sup>[61]</sup>. Remarkably, lost to follow-up was 20% more frequent in the children treated with lifestyle interventions than in children with drug treatment<sup>[61]</sup>. Similar disappointing findings concerning weight loss were observed in most pediatric obesity treatment centers<sup>[68]</sup>.

The high drop-out rate of medical care system may be caused by the characteristics of children, and adolescents with type 2 diabetes mellitus as well as their families (*e.g.*, lack of psychosocial support and parenting skills, lack of motivation for lifestyle changes, and inadvertent constraints to therapy adherence), but also by lacking efficacy and/or quality of medical care. Evaluation studies are urgently needed to analyze the reasons why children with type 2 diabetes mellitus are frequently lost-to follow-up.

## OPTIMIZING COMPLIANCE IN CHILDREN AND ADOLESCENTS WITH TYPE 2 DIABETES MELLITUS

Compliance is a major problem in those with lifestyle-related diseases such as type 2 diabetes mellitus<sup>[60]</sup>. To maximize compliance and treatment adherence services for children with type 2 diabetes mellitus will need to be multidisciplinary in nature, and incorporate specific skills in pediatrics, diabetes mellitus, nutrition, psychology, social work, and diabetes mellitus education<sup>[60]</sup>. It is questionable that the traditional techniques used to optimize glycemic control in patients with type 1 diabetes mellitus

are also useful in children with type 2 diabetes mellitus<sup>[60]</sup>. Furthermore, it is debatable whether the most appropriate context for therapy of type 2 diabetes mellitus in adolescents is a tertiary pediatric hospital and yet traditionally, this is where most are referred<sup>[60]</sup>. Given the very strong family context, this is one area where an all-of-family approach led by a family doctor may increase compliance since the whole family can be involved in treatment<sup>[60]</sup>.

## PSYCHOSOCIAL ASPECTS IN CHILDREN AND ADOLESCENTS WITH TYPE 2 DIABETES MELLITUS

Adolescents with type 2 diabetes mellitus rate lower "Quality of Life" scores than their peers with type 1 diabetes mellitus<sup>[69]</sup> and the burden of neuropsychiatric disorders in young people with type 2 diabetes mellitus is high, with as many as one in five experiencing either psychiatric illness or neurodevelopmental/behavioral problems<sup>[70]</sup>. Coupled with this, young people with type 2 diabetes mellitus are often from lower socio-economic backgrounds where social networks are less well developed to cater for the psychosocial needs of the individual<sup>[60]</sup>. This is important as psychosocial factors represent a significant barrier to optimum self-management in adolescents with type 2 diabetes mellitus<sup>[71]</sup>, often leading to a vicious circle of spiraling poor self-management and increasing psychosocial problems<sup>[60]</sup>.

Intiguing evidence from adult studies also suggests that daily negative mood has an adverse effect on fasting glucose concentrations on the following day<sup>[72]</sup> suggesting that poor psychosocial health may also be physiologically related to poorer glycemic control<sup>[60]</sup>. Therefore psychologists should be core component of care of children and adolescents with type 2 diabetes mellitus<sup>[60]</sup>.

## PREVENTION OF TYPE 2 DIABETES MELLITUS IN CHILDREN AND ADOLESCENTS

The financial and societal consequences of the emerging epidemic of type 2 diabetes mellitus are substantial and demand an urgent public health response. Emphasis must be placed upon preventive behaviors and early detection. Prevention of type 2 diabetes mellitus means prevention of obesity in childhood. The effect of weight loss on comorbid conditions and, most importantly, on the development of type 2 diabetes mellitus has been unequivocally proven<sup>[58,73]</sup>. As prevention should start very early in life, perhaps even before birth, a population and community approach for prevention of obesity in childhood and hence type 2 diabetes mellitus in childhood and adolescence seems to be the most promising and reasonable treatment strategy available at present.

However, primary prevention has proven to be difficult or impossible in most societies<sup>[74]</sup>. A multidisciplinary team approach is needed to develop and secure

preventive strategies. Good nutrition and modest exercise for pregnant women as well as monitoring of intra-uterine growth of the foetus are mandatory. After birth, rapid weight gain should be avoided and the principles of good nutrition and physical activity be taught at all ages. Breast-feeding should be strongly recommended. Children's food choice can be influenced by early intervention and guidance. In fact, teacher training, modification of school meals and physical education are effective in reducing risk factors for obesity<sup>[16]</sup>.

The cost-effectiveness of group and mixed family-based treatments for childhood obesity has been tested and proven for motivated families<sup>[58]</sup>. Therefore, family-based, behavioural treatment for obesity is also effective in preventing type 2 diabetes mellitus and is also extremely cost-effective. However, in unmotivated families, treatment remains difficult and frustrating for the patients and his family, as well as for the multidisciplinary team caring for the obese child.

To prevent the development of type 2 diabetes mellitus and its life-shortening sequelae early detection of impaired glucose regulation may represent an appropriate strategy to prevent type 2 diabetes mellitus, as subjects with impaired glucose tolerance are at increased risk of developing this disease<sup>[75]</sup>. Recent intervention studies have convincingly demonstrated that adoption of a healthy lifestyle characterized by healthy eating, regular physical activity and subsequent modest weight loss can prevent the progression of impaired glucose tolerance to clinical diabetes mellitus<sup>[76]</sup>. However, the use metformin was not effective to prevent type 2 diabetes mellitus in obese adolescents with impaired glucose tolerance<sup>[77]</sup>. This might be attributed to the fact, that impaired glucose tolerance normalized physiologically at the end of puberty even without intervention<sup>[20]</sup>.

## CONCLUSION

Type 2 diabetes mellitus is still rare in childhood and adolescence, but recent reports indicate an increasing prevalence around the world possibly due to increasing prevalence of obesity in children and adolescents. This is particularly the case in the United States but has also been reported in other countries in Asian and Europe. It is becoming increasingly clear that obese children and adolescents with clinical signs of insulin resistance (acanthosis nigricans, dyslipidemia, hypertension, PCOS) or relatives with type 2 diabetes mellitus or of particular ethnic populations (Asian, American Indian, African-Americans, Hispanics) above the age of 10 years should be screened for type 2 diabetes mellitus. Prevention and treatment of type 2 diabetes mellitus should become one of the prime targets of public health intervention programs. Much more attention should be given to the prevention and development of preventive strategies early in life. Finally, and most importantly, public awareness of the increasing health burden and economic dimension of the childhood obesity epidemic is of importance. Physicians should make the public aware of both the

childhood obesity epidemic and its serious consequences, not least type 2 diabetes mellitus.

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WJD 5<sup>th</sup> Anniversary Special Issues (2): Type 2 diabetes**Therapeutic effects of sericin on diabetic keratopathy in Otsuka Long-Evans Tokushima Fatty rats**

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**Abstract**

An Otsuka Long-Evans Tokushima Fatty (OLETF) rat provides a useful model for studies to develop corneal wound healing drugs for use in diabetic keratopathy resulting from type 2 diabetes mellitus. We investigated the effects of sericin on corneal wound healing in OLETF rats. Corneal wounds were prepared by removal of the corneal epithelium and documented using a TRC-50X. Sericin was instilled into the eyes of rats five times a day following corneal abrasion. The plasma levels of glucose, triglycerides, cholesterol and insulin in 38 wk old OLETF rats were significantly higher than in normal control rats (LETO rats), and the rate of corneal wound healing in OLETF rats was slower than in normal rat, probably due to the suppression of cell migration and proliferation caused by high plasma glucose levels. The corneal wounds of OLETF rats instilled with saline showed almost complete healing 72 h after corneal epithelial abrasion. On the other hand, the instillation of sericin has a potent effect in promoting wound healing and wound size reduction in OLETF rats and the wounds showed almost complete healing at 48 h after abrasion. The sericin may be an effective and safe drug to promote corneal wound healing in diabetic keratopathy.

reserved.

**Key words:** Sericin; Diabetic keratopathy; Cornea; Type 2 diabetes mellitus; Otsuka Long-Evans Tokushima Fatty rat**Core tip:** Otsuka Long-Evans Tokushima Fatty (OLETF) rats provide a useful model for studies to develop corneal wound healing drugs for use in diabetic keratopathy resulting from type 2 diabetes mellitus. The present study demonstrates that the rate of corneal wound healing in OLETF rats is slower than in normal rat, probably due to the suppression of cell migration and proliferation caused by high plasma glucose levels. The instillation of sericin has a potent effect in promoting wound healing and wound size reduction in OLETF rats. The sericin may be an effective and safe drug to promote corneal wound healing in diabetic keratopathy.

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**INTRODUCTION**

Diabetes mellitus is a common metabolic disorder, affecting more than 190 million people worldwide<sup>[1,2]</sup>. The prevalence of type 2 diabetes mellitus is increasing rapidly, affecting the health of millions of humans and will continue to do so in the near future. Among the factors responsible for the increasing prevalence of this disease are obesity, the consumption of energy-dense diets and low levels of physical activity<sup>[3]</sup>. The development of type 2 diabetes mellitus is associated with pancreatic  $\beta$ -cells dysfunction occurring together with insulin resistance.

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Normal  $\beta$ -cells can compensate for insulin resistance by increasing insulin secretion<sup>[4]</sup> but insufficient compensation leads to the onset of glucose intolerance. Once hyperglycemia becomes apparent,  $\beta$ -cell function progressively deteriorates, glucose-induced insulin secretion becomes further impaired and degranulation of  $\beta$ -cells becomes evident, often accompanied by a decrease in the number of  $\beta$ -cells<sup>[5-8]</sup>. Recently, it was reported that the production of reactive oxygen species (ROS) mediated by glycation reduces insulin gene transcription and decreases the number of  $\beta$ -cells through apoptosis<sup>[9,10]</sup>. Once  $\beta$ -cells are exposed to ROS under diabetic conditions they are likely to be profoundly damaged due to their low levels of antioxidant enzyme expression<sup>[11]</sup>.

Ocular complications secondary to type 2 diabetes mellitus are a well known cause of diabetic keratopathy<sup>[12]</sup>. Diabetic keratopathy is an entity that includes slow healing or loose adhesion of the corneal epithelium after wounding in diabetic patients. Histologically, it involves a thickening of the corneal epithelial basement membrane and morphological changes in the corneal epithelium and endothelium<sup>[13-18]</sup>. Clinically, the damage to the corneal epithelium during vitreous surgery and retinal photocoagulation sometimes induces vision-threatening corneal complications, such as persistent epithelial defects in diabetic patients<sup>[19]</sup>. It has been reported that such diabetic keratopathy is experienced by 50% or more of diabetic patients<sup>[20]</sup>.

The corneal wound repair process involves cell adhesion, migration, proliferation, matrix deposition and tissue remodeling<sup>[21]</sup>. Many of these biological processes are mediated by growth factors, cytokines and other mediators released in injured tissues or cells<sup>[22]</sup>. These growth factors have been recognized as important mediators of proper wound repair<sup>[23]</sup>, and treatment with growth factors such as platelet-derived growth factor-BB, recombinant human epidermal growth factor and fibronectin has been shown to be beneficial for patients with chronic pressure ulcers or non-healing diabetic ulcers<sup>[24-30]</sup>. However, these autologous serum eye drops have problems in terms safety and stability. In addition, for reasons of effectiveness, safety and stable supply, a potent wound healing agent for human corneal wounds has not yet been introduced. Therefore, a potent corneal wound healing agent for human corneal wounds that avoids these problems is highly anticipated. In this study, we attempted the development of corneal wound healing drugs against type 2 diabetes mellitus.

## MODEL FOR STUDY OF TYPE 2 DIABETES MELLITUS

In the development of corneal wound healing drugs against type 2 diabetes mellitus, the selection of the experimental animal is very important. Animal models used to investigate diabetes mellitus include streptozotocin-induced diabetic rats as an animal model for insulin-dependent diabetes mellitus<sup>[31,32]</sup>. Although, hyperglycemia

is present in streptozotocin-induced diabetic rats, the general pathophysiology of these models differs from that of non-insulin-dependent diabetes mellitus in humans. On the other hand, the general pathophysiology of Goto-Kakizaki (GK) rats, which are used as a model animal for type 2 diabetes mellitus, is similar<sup>[33]</sup>. However, GK rats develop type 2 diabetes mellitus without metabolic syndrome. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an established model of human type 2 diabetes<sup>[34]</sup>. Nearly 100% of male OLETF rats develop a diabetic syndrome by 25 wk of age; hyperglycemia and hyperinsulinemia are exhibited in the early phases of the disease as a result of islet cell hyperplasia and peripheral insulin resistance<sup>[35-38]</sup>, and a close relationship was observed between the plasma glucose and insulin levels ( $y = -51x + 335$ ,  $r = -0.9094$ ) in 38 wk old OLETF rats (Table 1)<sup>[39]</sup>. With continued aging, the rats eventually develop hypoinsulinemia as a result of the deterioration of islet beta cells (Table 1)<sup>[36,38,39]</sup>. These changes are similar to those in GK rats, which are used as a model animal for type 2 diabetes mellitus<sup>[40]</sup>. In addition, OLETF rats develop type 2 diabetes mellitus *via* a metabolic syndrome. Therefore, the changes in the biological characteristics of OLETF rats show an obvious correspondence to those that take place in human type 2 diabetes mellitus, indicating that OLETF rats provide a better model than GK rats for studies to clarify the effects of corneal wound healing drugs for type 2 diabetes mellitus<sup>[39]</sup>.

## KINETIC ANALYSIS OF THE RATE OF CORNEAL WOUND HEALING

The maintenance of corneal epithelial cell mass can be viewed as the result of three separate, independent phenomena<sup>[41]</sup>. Thoft and Friend have termed these: X, the proliferation of basal epithelial cells; Y, the contribution to the cell mass of the centripetal migration of peripheral cells; and Z, epithelial cell loss from the surface. Corneal epithelial maintenance thus can be defined by the equation  $X + Y = Z$ , which simply states that if the corneal epithelium is to be maintained, cell loss must be balanced by cell replacement<sup>[42]</sup>. The corneal wound healing process is divided into three sequential and partially overlapping steps: epithelial cell loss from the surface (Z) reduces and eventually covers the wound surface (Y), while cell proliferation (X) provides cells to rebuild the tissue and tissue remodeling to restore the stratified epithelium<sup>[33,43-48]</sup>. Therefore, it is important to develop the evaluation method of cell proliferation (X) and migration (Y) in the study for corneal wound healing using rat debrided corneal epithelium.

It has been reported that the early stages of epithelial wound closure rely predominately on cell migration rather than cell proliferation; cell proliferation starts approximately 24 h after corneal epithelial injury, after which tissue remodeling to restore the stratified epithelium occurs<sup>[49,50]</sup>. Furthermore, Zagon *et al.*<sup>[49]</sup> showed that

**Table 1** Body weight and some blood test values for diabetes mellitus in 38 and 60 wk old normal and Otsuka Long-Evans Tokushima Fatty rats

	38 wk old		60 wk old	
	Normal	OLETF	Normal	OLETF
Weight (g)	488.6 ± 14.2	621.3 ± 19.7 <sup>a</sup>	526.3 ± 33.0	416.3 ± 17.4 <sup>c</sup>
Glucose (mg/dL)	119.3 ± 4.9	213.5 ± 15.7 <sup>a</sup>	140.8 ± 3.6	244.3 ± 23.9 <sup>c</sup>
Triglycerides (mg/dL)	128.0 ± 9.3	419.8 ± 22.2 <sup>a</sup>	150.0 ± 14.4	335.8 ± 11.0 <sup>c</sup>
Total cholesterol (mg/dL)	101.4 ± 11.4	209.2 ± 11.1 <sup>a</sup>	83.6 ± 14.3	274.5 ± 26.2 <sup>c</sup>
Insulin (ng/dL)	105.5 ± 11.6	237.4 ± 26.6 <sup>a</sup>	111.1 ± 6.7	83.0 ± 7.2 <sup>c</sup>

The data are presented as mean ± SE of 4 independent rats. <sup>a</sup>*P* < 0.05 vs 38 wk old normal rats for each category; <sup>c</sup>*P* < 0.05 vs 60 wk old normal rats for each category. OLETF: Otsuka Long-Evans Tokushima Fatty.

the corneal wounds of Sprague-Dawley rats (250-300 g) were covered due to cell migration by 24 h after corneal epithelial injury. After that, the interior damage was healed by cell proliferation<sup>[49,51]</sup>. On the other hand, the wound surfaces in 38 wk old normal and OLETF rats were not completely covered by cell migration 24 h after corneal epithelial injury. Corneal wound healing in 38 wk old OLETF rats takes place in two clear phases. The second phase of healing, involving cell migration and proliferation, may be the source of the delay in corneal wound healing in the 38 wk old OLETF rats. Therefore, we analyzed the two phases of corneal wound healing in 38 wk old normal and OLETF rats by kinetic analysis. The rate of corneal wound healing is represented by the corneal wound healing rate constant ( $\alpha$  and  $\beta$ , /h) and the corneal wound healing rate constant was calculated from the following equations (Eq.1 and Eq.2) and the iterative nonlinear least-squares regression procedure MULTI<sup>[52]</sup>.

$$\text{Corneal wound (\%)} = \text{wound area}_{0-72\text{h}} / \text{wound area}_{0\text{h}} \times 100 \quad \text{Eq.1}$$

$$W_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \quad \text{Eq.2}$$

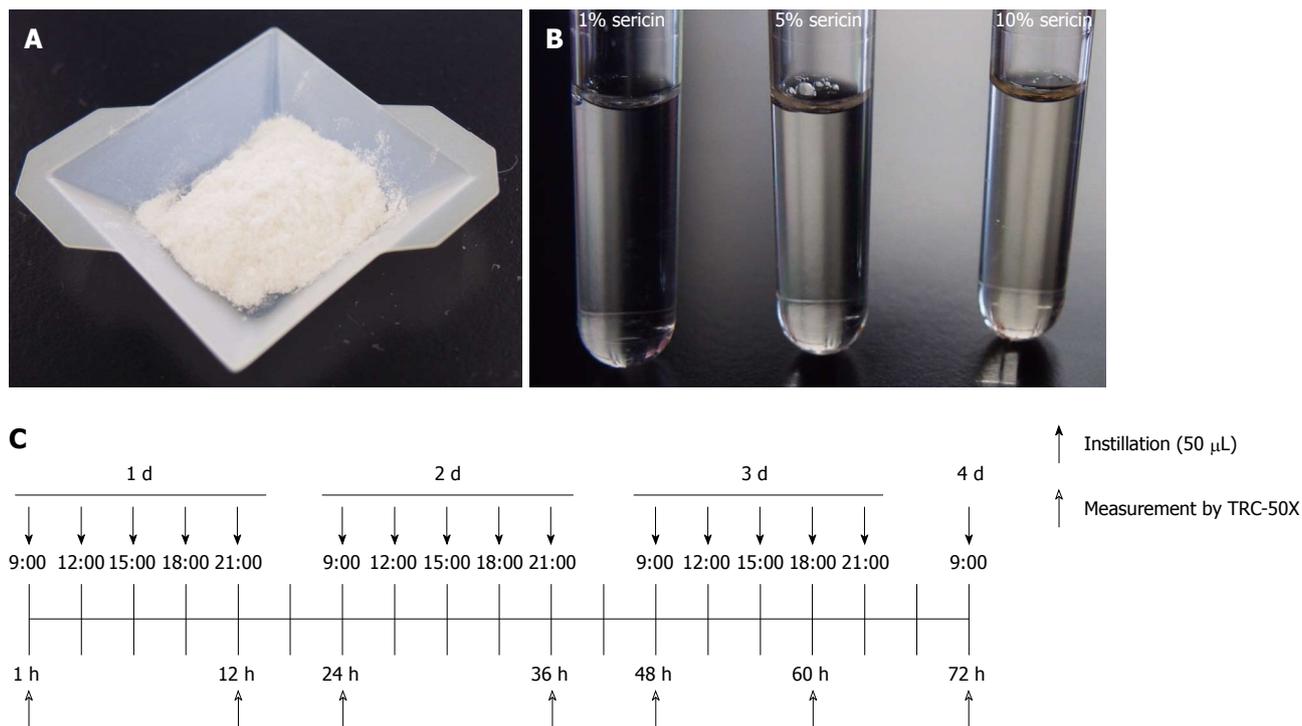
where *t* is time (0-72 h) after corneal abrasion and  $W_t$  is the percentage of corneal wound (%) at the corresponding time.  $W_0$  is the percentage of corneal wound (%) at time 0.  $\alpha$  and  $\beta$  show the corneal wound healing rate constants in the first and second phases, respectively. *A* and *B* are the corneal wound areas (%) in the  $\alpha$ - and  $\beta$ -phases, respectively. In addition, the  $\alpha$  represents cell migration, which is the main wound healing process up to 24 h after corneal epithelial abrasion, while the  $\beta$ -phase, which takes place 18-72 h after corneal epithelial abrasion, represents cell proliferation<sup>[52]</sup>. The  $\beta$  values for 38 wk old OLETF rats were significantly lower than those for normal rats at corresponding ages. The contribution ratio *A* of the corneal wound healing process to the  $\beta$ -phase in 38 wk old normal rats was significantly higher than the ratio *B* of the corneal wound healing process for the second phase. The contribution ratio *B* of the corneal wound healing process for the  $\beta$ -phase in 38 wk old OLETF rats tended to increase. Therefore, a deficit in cell proliferation is predominantly responsible for the delay in corneal wound healing in this model (OLETF rat). On the other hand, it was known that corneal wound healing in normal rats was delayed with

aging, and the corneal wound healing rate constant of 38 wk old normal rats was lower than that of 7 wk old normal rats. The  $\alpha$  and  $\beta$  values for 38 wk old normal rats were similar and the contribution to the corneal wound healing process of ratio *A* for the  $\beta$ -phase is significantly higher than the *B* for the  $\beta$ -phase. This result suggests that the corneal wounds of old normal rats are repaired by both cell migration and proliferation, and the rate of healing wound becomes equivalent once cell proliferation begins<sup>[52]</sup>.

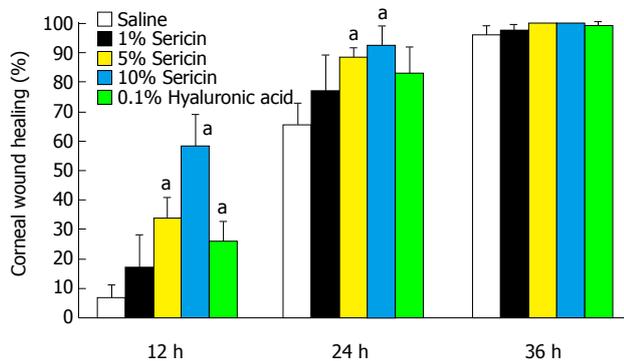
It is important to understand the mechanisms underlying the delay in corneal wound healing in type 2 diabetes mellitus. In diabetes, the levels of glucose in the cornea and tears are increased. Glucose levels in the corneal epithelium have been reported to be 6-fold higher (1.8 to 12.2  $\mu\text{mol/g dw}$ ) in diabetic patients than in normal controls<sup>[53]</sup> and large increases in the glucose content of tears (range 2.16-9.55 mg/dL and 14.69-27.02 mg/dL for normal and diabetic patients, respectively) have also been reported<sup>[54-56]</sup>. March *et al.*<sup>[57]</sup> reported that the glucose content of tears is approximately 10% the plasma glucose level and that the glucose content of tears follows changes in plasma glucose levels. High glucose levels suppress the cellular behavior (cell migration and proliferation) of human corneal epithelial cells<sup>[58]</sup>. In addition, it has been reported that the instillation of insulin normalizes the delay in corneal wound healing in streptozotocin rats<sup>[20]</sup>. Furthermore, a close relationship was observed between the  $\alpha$  and  $\beta$  values and glucose levels in 38 wk old OLETF rats, unlike the progression of type 2 diabetes mellitus<sup>[52]</sup>. These reports indicate that the decrease in corneal wound healing in diabetic keratopathy is caused by a suppression of cell migration and proliferation due to high glucose levels in tears. In addition, this result supports the previous findings for human diabetic keratopathy<sup>[58]</sup>.

## HEALING EFFECT OF SERICIN ON DIABETIC KERATOPATHY

Proteins such as fibroin and sericin are the main constituents of silk, with fibroin contributing 70% to 80% and sericin 20% to 30% of the total cocoon weight<sup>[59]</sup>. When cocoons or raw silk are used for textiles, the sericin is mostly removed from the cocoon and disposed of



**Figure 1** Picture of sericin and protocol in this study. A: Picture of sericin; B: Picture of sericin solution. The sericin solutions used in this study were prepared by adding sericin to saline (pH 6.5-7.5); C: Protocol for instillation of sericin. Saline, sericin or hyaluronic acid solutions were instilled into the eyes of rats five times a day.

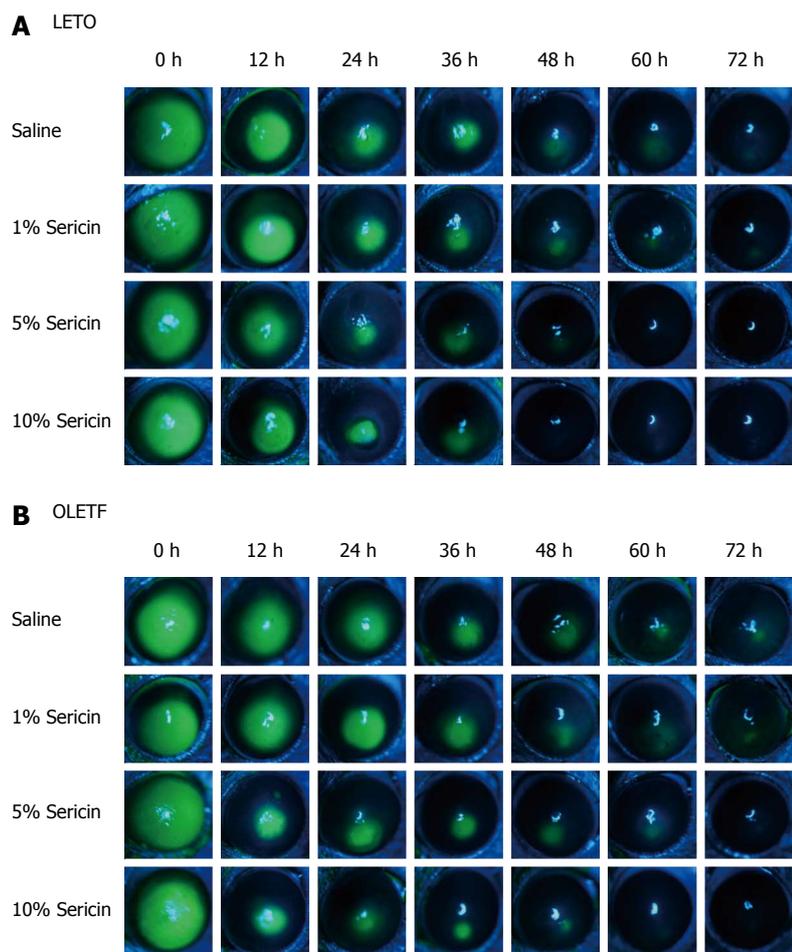


**Figure 2** Corneal images of Wistar rats with or without the instillation of sericin solutions. The corneal epithelium was removed with a BD Micro-Sharp™ and the resulting corneal wounds were dyed with 1% fluorescein solution. Saline, sericin or hyaluronic acid solutions were instilled into the eyes of rats five times a day. <sup>a</sup>*P* < 0.05 vs saline-instilled rat.

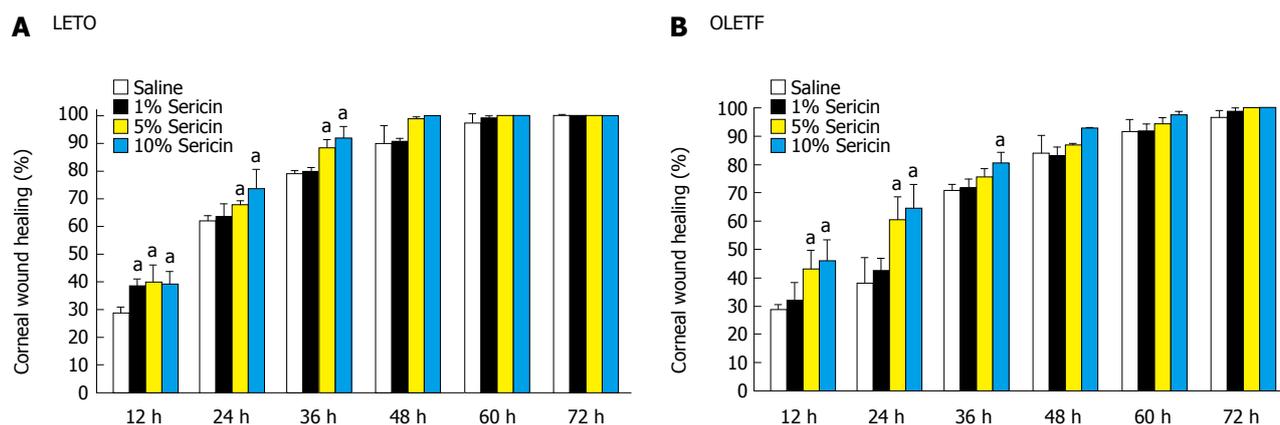
unused. However, sericin has recently been investigated for its activities in biotechnological fields (Figure 1A)<sup>[60,61]</sup>. Terada *et al.*<sup>[62]</sup> found growth promotion in several human cell lines and mouse hybridomas when sericin was added to the culture media. It is possible that sericin may be applied as eye drops for corneal wound repair. The sericin solutions used in this study were prepared by adding sericin to saline (pH 6.5-7.5, Figure 1) and the instillation of 10% sericin (30 kDa, Seiren Co, Ltd, Fukui, Japan) produced no observable neovascularization or inflammation<sup>[51]</sup>. In addition, the instillation of 10% sericin resulted in a significantly greater rate of corneal wound size reduction and healing than the instillation of 0.1%

hyaluronic acid in Wistar rat eyes, probably by increasing cell migration and proliferation (Figure 2)<sup>[51]</sup>. In this study, the rates of corneal wound healing in both Long-Evans Tokushima Otsuka rats (LETO rat, normal control) and OLETF rat eyes were faster following the instillation of sericin than in the case of saline instillation, and the rate constant increased with increasing sericin concentration. The rates of corneal wound healing in OLETF rat eyes instilled with 5% or 10% sericin were similar to those of LETO rats treated similarly. In addition, the instillation of sericin did not affect glucose levels in the OLETF rats (saline instillation, 213.0 ± 19.7 mg/dL; sericin instillation, 221.1 ± 13.9 mg/dL; mean ± SE of 4 independent OLETF rats). The instillation of 5% or 10% sericin increased the corneal wound healing in OLETF rats 12 h after corneal epithelial abrasion (Figures 3 and 4). Furthermore, we reported that sericin increases the adhesion and proliferation<sup>[51]</sup>. Taken together, the instillation of sericin solution counters the decreases in the rates of cell migration and proliferation, thus preventing the delay in corneal wound healing in OLETF rats (Figure 5).

Next, we investigated the effects of the sericin on cell adhesion and proliferation in a human cornea epithelial cell line (HCE-T) since the effect of sericin in corneal wound healing is a result from the increase in cell migration and proliferation. The adhesion and proliferation of HCE-T cells reached a maximum when treated with 0.1%-0.2% sericin solution; the levels of adhesion and proliferation of 1.0% sericin-treated HCE-T cells did not differ significantly from those of control HCE-T cells (Figure 6). On the other hand, the instillation of



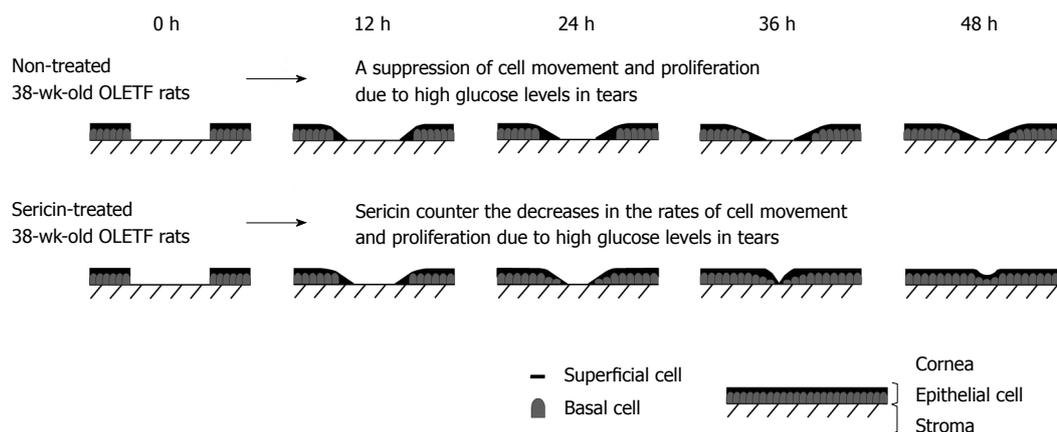
**Figure 3 Corneal images.** A: Long-Evans Tokushima Otsuka (LETO) rats; B: Otsuka Long-Evans Tokushima Fatty (OLETF) rats with or without the instillation of sericin solutions. The photograph was reported in reference 39. The corneal epithelium was removed with a BD Micro-Sharp™ and the resulting corneal wounds were dyed with 1% fluorescein solution. Saline or sericin solutions were instilled into the eyes of rats five times a day.



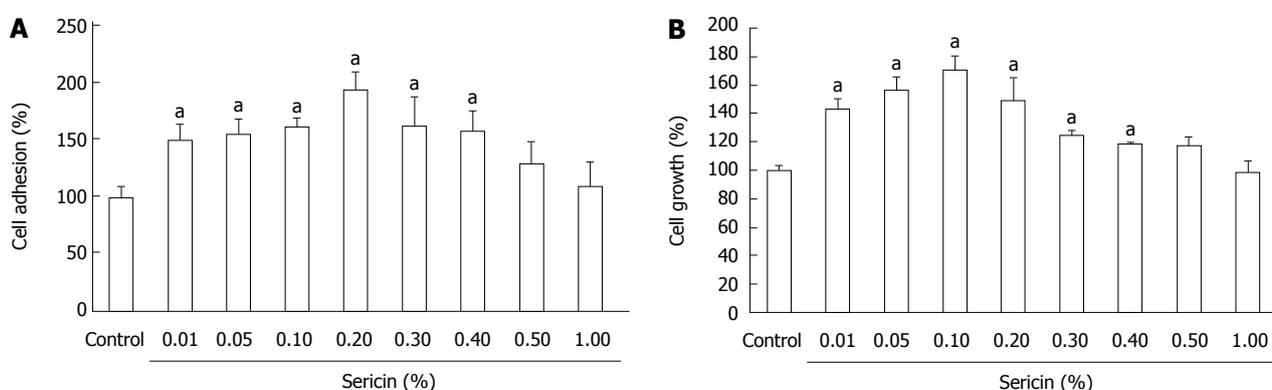
**Figure 4 Effect of sericin solutions on corneal wound healing.** A: Long-Evans Tokushima Otsuka (LETO) rats; B: Otsuka Long-Evans Tokushima Fatty (OLETF) rat eyes. The data were reported in reference 39. Saline or sericin solutions were instilled into the eyes of rats five times a day. The data are presented as mean ± SE of 3-5 independent rat corneas. <sup>a</sup>*P* < 0.05 vs saline-instilled rat.

high concentration sericin solutions (1%-10%) promoted enhanced wound healing in the corneal wound rat model. It is known that the concentration of drugs administered in eye drops is diluted to approximately 20% by lacrimal fluids and that the components of eye

drops are excreted though the nasolacrimal duct into the mouth<sup>[34]</sup>. Thus, our findings suggest that the optimum concentration of the sericin solutions in the *in vivo* instillation experiment, which involves a short residence time, is higher than in the *in vitro* experiment. These findings



**Figure 5** The function of cell migration and proliferation in corneal wound healing in 38 wk old Otsuka Long-Evans Tokushima Fatty rats with or without the instillation of sericin solutions. The movement of superficial cells shows cell migration and the number of basal cells represents cell proliferation. OLETF: Otsuka Long-Evans Tokushima Fatty.



**Figure 6** Effect of sericin on the adhesion (A) and growth (B) of human cornea epithelial cell line. The data were reported in reference 51. Human cornea epithelial cell line (HCE-T) cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 containing 5% (v/v) heat-inactivated fetal bovine serum, 0.1 mg/mL streptomycin and 1000 IU/mL penicillin. Cell growth was calculated by TetraColor One. The amount of cell adhesion and growth were represented by the following equation: cell adhesion or growth (%) =  $Ab_{Sericin\ treatment} / Ab_{Scontrol} \times 100$ . The data are presented as mean  $\pm$  SE of 5-25 experiments. \* $P < 0.05$  vs control HCE-T cells.

provide information significant for designing further studies to develop potent drugs to improve the corneal wound healing ability of diabetic patients.

## CONCLUSION

OLETF rats provide a useful model for studies to develop corneal wound healing drugs for use in diabetic keratopathy resulting from type 2 diabetes mellitus. The present study demonstrates that the rate of corneal wound healing in OLETF rats is slower than in LETO rats, probably due to the suppression of cell migration and proliferation caused by high plasma glucose levels. The instillation of sericin solution has a potent effect in promoting wound healing and wound size reduction in LETO and OLETF rats. The sericin may be an effective and safe drug to promote corneal wound healing in diabetic keratopathy.

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## Classification of diabetic retinopathy and diabetic macular edema

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### Abstract

The global incidence and prevalence of diabetes mellitus (DM) have reached epidemic proportions. Estimates indicate that more than 360 million people will be affected by DM by 2030. All of these individuals will be at risk of developing diabetic retinopathy (DR). It is extremely important to categorize, classify and stage the severity of DR in order to establish adequate therapy. With proper management more than 90% of cases of visual loss can be prevented. The purpose of the current paper is to review the classification of DR with a special emphasis on the International Clinical Disease Severity Scale for DR. This new classification is simple to use, easy to remember and based on scientific evidence. It does not require specialized examinations such as optical coherence tomography or fluorescein angiography. It is based on clinical examination and applying the Early Treatment of

Diabetic Retinopathy Study 4:2:1 rule.

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**Key words:** Diabetic retinopathy classification; Diabetic macular edema classification; International clinical disease severity scale; Diabetic macular edema; Diabetic retinopathy

**Core tip:** The International Clinical Disease Severity Scale for diabetic retinopathy has been proposed to facilitate communication between diabetic care givers. This new classification is simple to use, easy to remember and based on scientific evidence. It does not require specialized examinations such as optical coherence tomography or fluorescein angiography. It is based on clinical examination and applying the Early Treatment of Diabetic Retinopathy Study 4:2:1 rule.

Wu L, Fernandez-Loaiza P, Sauma J, Hernandez-Bogantes E, Masis M. Classification of diabetic retinopathy and diabetic macular edema. *World J Diabetes* 2013; 4(6): 290-294 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/290.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.290>

### INTRODUCTION

Currently the world faces a challenge of epidemic proportions called diabetes mellitus (DM). According to estimates of the World Health Organization, the number of people worldwide with DM will rise to 360 million by the year 2030<sup>[1]</sup>. DM is no longer a disease of rich developed countries. Changes in dietary habits, obesity and physical inactivity are responsible for spreading this epidemic into the developing countries. All individuals with DM will be at risk of developing diabetic retinopathy (DR).

DR is a progressive condition with microvascular al-

terations that lead to retinal ischemia, retinal permeability, retinal neovascularization and macular edema<sup>[2,3]</sup>. If left untreated patients with DR can suffer severe visual loss<sup>[4]</sup>. In developed countries DR constitutes the leading cause of blindness in the working age population<sup>[5]</sup> and has a considerable economic impact on society especially on healthcare systems<sup>[6-8]</sup>. Given that proper management of patients with DR can prevent more than 90% of cases of visual loss, it is extremely important to categorize, classify and stage the severity of DR in order to establish adequate therapy<sup>[9]</sup>. In particular, in diseases like DM where a multidisciplinary approach is needed, communication among colleagues of different medical specialties is crucial. It is imperative that retina specialists, general ophthalmologists, internists, and endocrinologists learn to speak the same language in order to provide patients with the care they deserve. The purpose of the current paper is to review the classification of DR with a special emphasis on the International Clinical Disease Severity Scale for DR.

## MODIFIED AIRLIE HOUSE CLASSIFICATION

In 1968 a group of experts met in Airlie House, Virginia to discuss what was known at the time about DR. An important outcome of that symposium was the development of a standardized classification of DR<sup>[10]</sup>. This classification was modified and used in the Diabetic Retinopathy Study (DRS). Briefly, it consisted of comparing stereophotographs in 7 standard photographic fields with the patient's findings in those same 7 photographic fields<sup>[11]</sup>. This same classification was modified for use in the Early Treatment of Diabetic Retinopathy Study (ETDRS). It became the gold standard for many years. The modified Airlie House Classification of DR is based on grading of stereophotographs of 7 fields and classifies DR into 13 complex levels ranging from level 10 (absence of retinopathy) to level 85 (severe vitreous hemorrhage or retinal detachment involving the macula)<sup>[12]</sup>. It is an excellent tool in the research setting but its clinical applicability is limited due to its complexity. Most ophthalmologists do not use this classification in their daily clinical work.

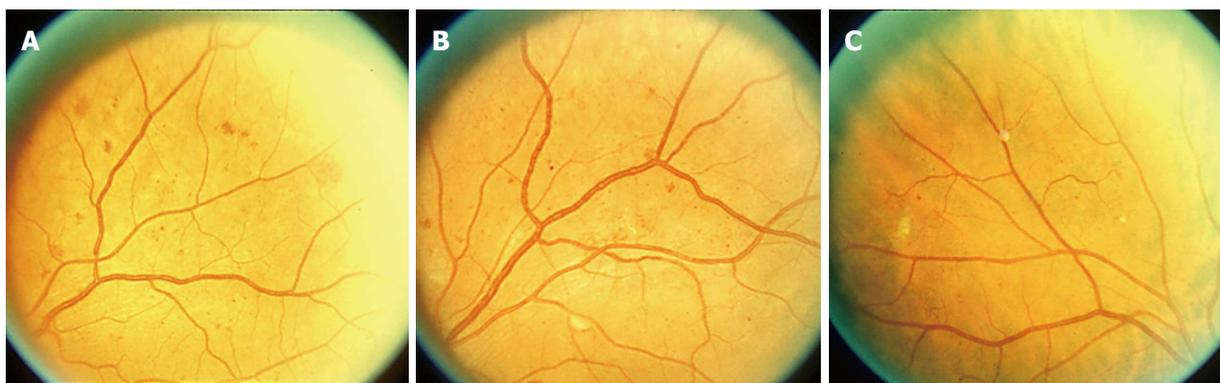
The ETDRS introduced the term clinically significant macular edema (CSME). CSME was defined upon slit lamp biomicroscopy as “(1) thickening of the retina at or within 500  $\mu\text{m}$  of the center of the macula; or (2) hard exudate at or within 500  $\mu\text{m}$  of the center of the macula associated with thickening of adjacent retina; or (3) a zone of retinal thickening 1 disc area or larger, any part of which is within 1 disc diameter of the center of the macula”<sup>[12]</sup>. The ETDRS found that macular photocoagulation was effective in reducing visual loss from CSME.

Others have divided diabetic macular edema (DME) into focal and diffuse subtypes<sup>[13]</sup>. Focal DME was initially defined as edema caused by focal leakage of microaneurysms. Clusters of microaneurysms are usually sur-

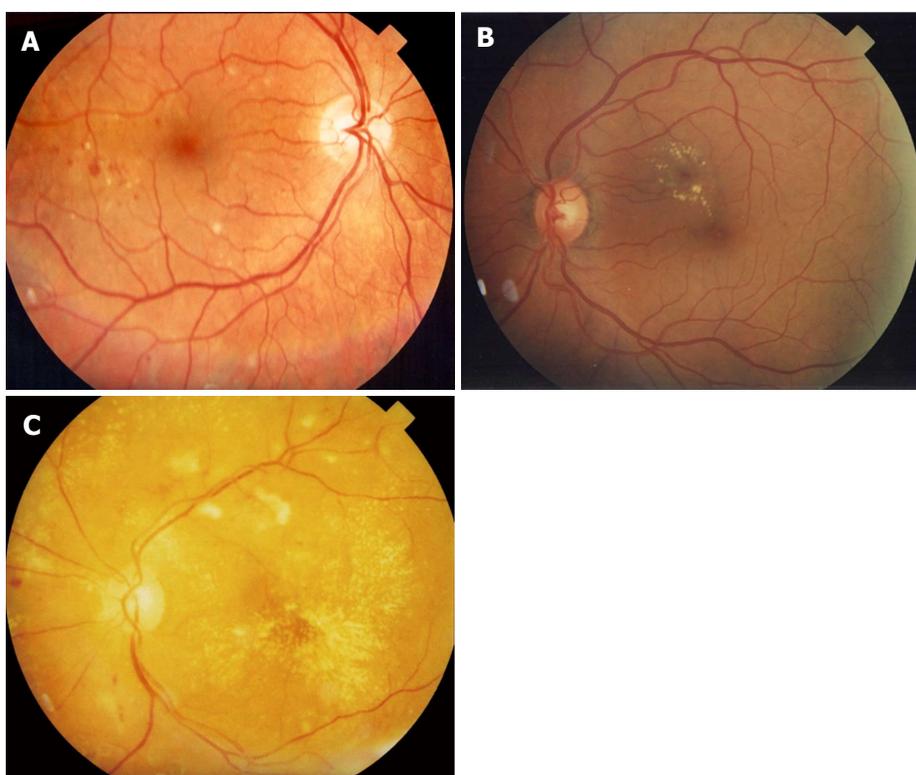
rounded by a circinate ring of hard exudate. In contrast, diffuse DME is caused by a generalized breakdown of the inner blood/retinal barrier. Microaneurysm, retinal capillaries and even arterioles contribute to the leakage. Hard exudates are usually not seen in diffuse DME<sup>[13]</sup>. As time went by, others included FA findings in their definition of diffuse or focal DME. In the ETDRS, the proportion of microaneurysmal fluorescein leakage was used to differentiate between focal and diffuse DME. Eyes with  $\geq 67\%$  of leakage originating from microaneurysms were classified as having focal DME. Eyes with microaneurysmal leakage between 33% and 66% were determined to have intermediate DME. Eyes with  $\leq 33\%$  microaneurysmal leakage were classified as having diffuse DME<sup>[14]</sup>. Others have also included OCT findings<sup>[15,16]</sup>. As Browning *et al.*<sup>[17]</sup> point out, the terms focal and diffuse DME have various definitions and mean different things to different people. Furthermore, since the terms do not seem to predict treatment outcomes they suggest that their use should be discouraged until an agreement is reached<sup>[17]</sup>.

## INTERNATIONAL CLINICAL DISEASE SEVERITY SCALE FOR DR

In an attempt to simplify the classification of DR, a number of experts met and created the International Clinical Disease Severity Scale for DR<sup>[18]</sup>. This disease severity scale is based upon the findings of the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) and the ETDRS. This new classification is simple to use, easy to remember and based on scientific evidence. There are five stages that are recognized. The first is “no apparent retinopathy”. As the name implies there are no diabetic fundus changes. The second stage is “mild non-proliferative retinopathy” (NPDR). This stage is characterized by the presence of a few microaneurysms. The third stage is “moderate NPDR” which is characterized by the presence of microaneurysms, intraretinal hemorrhages or venous beading that do not reach the severity of the standard photographs 2A (Figure 1A), 6A (Figure 1B) and 8A (Figure 1C). “Severe NPDR”, the fourth stage, is the key level to identify. Data from the ETDRS has shown that eyes in patients with DM type 2 that reach the grade of severe NPDR have a 50% chance of developing high risk characteristics if laser treatment is not instituted<sup>[19]</sup>. The diagnosis of severe NPDR is based on the 4:2:1 rule of the ETDRS<sup>[20]</sup>. Using standard photographs 2A, 6A and 8A to compare with the fundus findings, one can easily diagnose severe NPDR<sup>[20]</sup>. If hemorrhages of at least the magnitude of standard photograph 2A are present in all 4 quadrants, then by definition severe NPDR is present. If 2 quadrants or more have venous beading (VB) of the same magnitude or greater than standard photograph 6A, then by definition severe NPDR is present. If one or more quadrant has intraretinal microvascular abnormalities (IRMA) of the same magnitude or greater than standard photograph 8A, then by definition severe NPDR is



**Figure 1 Standard photograph.** A: 2A. Notice the intraretinal hemorrhages. If 4 quadrants have intraretinal hemorrhages of at least this magnitude then by definition severe non-proliferative retinopathy is present; B: 6A. Notice venous beading (VB). If 2 quadrants or more have VB of at least this magnitude then by definition severe non-proliferative retinopathy is present; C: 8A. Notice the intraretinal microvascular abnormalities (IRMA). If one or more quadrants has IRMA of at least this magnitude then by definition severe non-proliferative retinopathy is present.



**Figure 2 Diabetic macular edema.** A: Mild diabetic macular edema (DME). Notice that the hard exudates are located far from the center of the fovea; B: Moderate DME. Even though there is no thickening involving the center of the fovea, the hard exudates are threatening the center of the fovea; C: Severe DME. The center of the fovea is involved with hard exudate and thickening.

present. The final stage is “proliferative diabetic retinopathy” (PDR). PDR is characterized by neovascularization of the disc, neovascularization of the retina, neovascularization of the iris, neovascularization of the angle, vitreous hemorrhage or tractional retinal detachment. With regards to macular edema, it should be noted if macular edema is present or absent. If it is present then it can be further classified as mild, moderate and severe depending on the distance of the exudates and thickening from the center of the fovea<sup>[18]</sup> (Figure 2).

## FLUORESCEIN ANGIOGRAPHIC CLASSIFICATION

The investigators from the ETDRS recognized that some diabetic features could be assessed better with fluorescein angiograms (FA) than clinical fundus photographs. Thus the ETDRS also classified DR from FA. The ETDRS protocol consisted of stereoscopic FA of two 30° fields that extended along the horizontal meridian from 25° nasal to the disc to 20° temporal to the macula. Patient

findings were compared to standardized photographs. In the early-mid phase of the FA, the foveal avascular zone (FAZ), capillary loss, capillary dilatation, arteriolar and RPE abnormalities were assessed. Fluorescein leakage, fluorescein leakage source and cystoid changes were graded during the late FA phase<sup>[21]</sup>. This fluorescein angiographic classification scheme is time consuming, complex and ideal for the research setting but not for regular clinical use.

## OPTICAL COHERENCE TOMOGRAPHY CLASSIFICATION

Optical coherence tomography (OCT) is a non-invasive, noncontact transpupillary imaging modality that has revolutionized ophthalmic clinical practice. It utilizes light to image tissue using low coherence interferometry. OCT offers several advantages over slit lamp biomicroscopy and FA. OCT produces cross sectional images of the macula allowing objective evaluation of macular thickness and evaluation of the vitreomacular interface<sup>[22]</sup>. However, it must be noted that there is poor correlation between macular thickness and visual acuity<sup>[23]</sup>. The main disadvantage of OCT is the cost the required equipment, which limits its availability.

Several OCT patterns of morphological macular changes associated with DME have been described<sup>[16]</sup>. These include diffuse retinal thickening, cystoid macular edema, posterior hyaloidal traction, serous retinal detachment and tractional retinal detachment. These patterns are not exclusive of each other and may co-exist with one another. Despite attempts to implement an OCT-based classification of DME<sup>[24]</sup>, there has been no consensus on its use. A Cochrane review concluded that there is substantial disagreement between the OCT findings and CSME as defined by clinical examination<sup>[25]</sup>. Nevertheless the changing treatment paradigm of DME, where anti-VEGF agents are increasingly being used in the management of DME, demonstrates that OCT is an essential tool in the current management of this condition<sup>[26]</sup>.

## CONCLUSION

DR has a significant impact on the quality of life of patients with diabetes. The fact that a high percentage of visual loss can be prevented through laser, pharmacological or surgical treatment places great importance on the proper detection and classification of patients with DR especially those with severe NPDR who are at great risk for developing visual loss<sup>[19]</sup>. The International Clinical Disease Severity Scale has been developed<sup>[18]</sup>. This new classification is simple to use, easy to remember and based on scientific evidence. It does not require specialized examinations such as OCT or FA. It is based on clinical examination and applying the ETDRS 4:2:1 rule. Its use should be encouraged<sup>[27]</sup>. As new technologies for the evaluation of DR become available, current classification systems may be substituted or enhanced but the

basis of DR staging established by landmark clinical trials such as the ETDRS and DRS will most likely remain the same.

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## Clinical trials on corticosteroids for diabetic macular edema

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### Abstract

Diabetic macular edema (DME) is a common cause of visual impairment in diabetic patients. It is caused by an increase in the permeability of the perifoveal capillaries and a disruption of the blood retinal-barrier. The pathogenesis of DME is multifactorial. Several therapeutic modalities have been proposed for the treatment of DME. Corticosteroid treatments have emerged as an alternative therapy for persistent DME or refractory to conventional laser photocoagulation and other modalities, due to anti-inflammatory, anti-vascular endothelial growth factor and anti-proliferative effects. Many studies have demonstrated the beneficial therapeutic effect of corticosteroids with improvement to both retinal thickness and visual acuity in short-term on the treatment of DME. Peribulbar and intravitreal injections have been used to deliver steroids for DME with frequent injections due to the chronic and recurrent nature of the disease. Steroid-related side effects include elevated intraocular pressure, cataract, and injection related complications such as endophthalmitis, vitreous hemorrhage, and retinal detachment particularly with intravitreal steroid injections. In order to reduce the risks, complications and frequent dosing of intravitreal steroids, intravitreal implants have been developed recently to provide sustained release of corticosteroids

and reduce repeated intravitreal injections for the management of DME.

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**Key words:** Corticosteroids; Diabetic macular edema; Intravitreal triamcinolone acetonide injection; Intravitreal steroid sustained-release implants; Peribulbar steroid injections; Subtenon's steroid injections

**Core tip:** Despite the documented ocular side effects of corticosteroids by the time being they are still considered as one of the essential effective adjunct modalities for the treatment of diabetic macular edema especially in refractory and persistent cases.

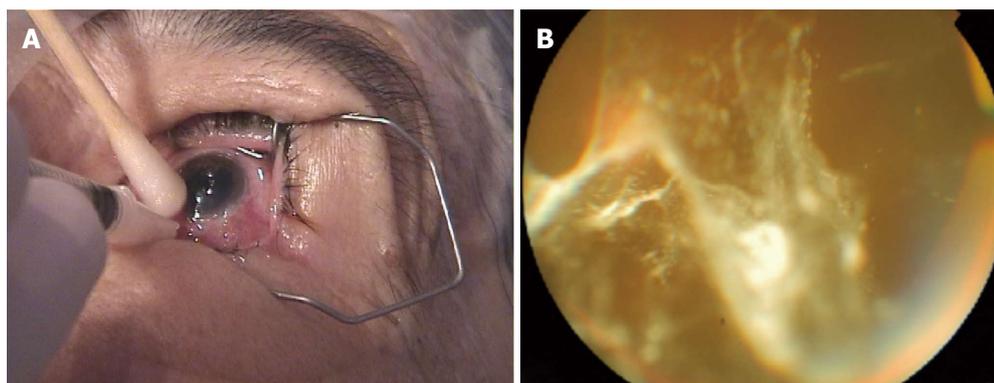
Al Dhibi HA, Arevalo JF. Clinical trials on corticosteroids for diabetic macular edema. *World J Diabetes* 2013; 4(6): 295-302 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/295.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.295>

### INTRODUCTION

Diabetic macular edema (DME) is considered to be a process of low-grade inflammation in which numerous inflammatory cells, mediators and cytokines are involved and subsequently lead to increase in vascular permeability<sup>[1-6]</sup>.

Corticosteroids are anti-inflammatory agents that stabilize retinal capillaries and tend to reduce their permeability by enhancing the activity or density of the tight junctions in the retinal capillary endothelium<sup>[6-8]</sup> as well inhibit and downregulate the metabolic pathway of the vascular endothelial growth factor<sup>[9-12]</sup> in order to decrease the leakage of plasma proteins into the interstitial tissue compartment and help to restore the osmotic gradient between blood and tissue compartments, and eventually will resolve edema formation<sup>[7,13,14]</sup>.

Corticosteroid has emerged as an alternative therapy for persistent DME or refractory to conventional laser



**Figure 1** Intravitreal triamcinolone acetonide injection. A: External aspect demonstrating intravitreal triamcinolone acetonide (IVTA) injection through the inferior pars plana in sterile conditions; B: Vitreous cavity aspect demonstrating IVTA injection with inferior deposition of triamcinolone acetonide crystals over the retinal surface on a patient with diabetic macular edema refractory to laser photocoagulation.

photocoagulation and other modalities<sup>[15-23]</sup>. Many studies have demonstrated the beneficial therapeutic effect of corticosteroids such as triamcinolone and dexamethasone on the treatment of diabetic macular edema<sup>[15-27]</sup>. Various routes have been used to deliver steroid for DME treatment including peribulbar injection, intravitreal injection and intravitreal implants.

## PERIBULBAR OR SUBTENON'S STEROID INJECTIONS

Peribulbar or subtenon's steroid injections have been used to treat diabetic macular edema either as monotherapy or as adjunctive therapy to laser<sup>[16,17,24,28]</sup>. Although they are not considered ideal to obtain a therapeutic dose of cortisone at the level of the retina<sup>[29,30]</sup>, short-term efficacy has been demonstrated with transient improvement to both retinal thickness and visual acuity but less effective than intravitreal therapy<sup>[16,17,30]</sup>. However, a phase II study sponsored by the national eye institute, showed no benefit in reducing retinal thickness by adding peribulbar steroids to focal laser treatment for eyes with mild DME and good visual acuity<sup>[6,24]</sup>. Although, subtenon's approach is clearly less invasive than the intravitreal one<sup>[31]</sup>, it is not free of potential complications such as accidental injection directly into the choroidal or retinal circulation, perforation of the ocular bulb, occlusion of the central retinal artery and cataract<sup>[31]</sup>. Other complications reported are blepharoptosis, orbital fat atrophy, strabismus and conjunctival necrosis<sup>[31,32]</sup>. It is important to add, that intra-ocular pressure (IOP) is not increased by the use of the posterior sub-Tenon's approach in comparison to anterior sub-Tenon or intravitreal triamcinolone acetonide (IVTA) injections with the exception of steroid responder patients, the risk of IOP elevation is 44% steroid responders compared to 13% in non steroid responders<sup>[28,31-34]</sup>.

## INTRAVITREAL TRIAMCINOLONE ACETONIDE INJECTION

Intravitreal triamcinolone acetonide (IVTA) injection

has been widely used to treat DME particularly diffuse macular edema that persist after appropriate laser treatment (Figures 1 and 2). Many clinical trials have been conducted to evaluate the efficacy of IVTA therapy for DME<sup>[18-23,35-52]</sup>. IVTA has shown significant improvements in diabetic macular edema and visual acuity in the short term and found to be transient. The therapeutic effect is typically seen within 1 wk and the duration of the effect increased with increasing dosage. However, in many patients re-injections are needed every three to six months as the effect diminishes<sup>[18,19,42,44,53-57]</sup>.

The Diabetic Retinopathy Clinical Research Network (DRCR) compared 2 doses of IVTA as monotherapy to focal/grid laser photocoagulation in 840 eyes with DME. The 4-mg IVTA group had better visual acuity at four months; however, at 16 mo, two years and three years, the laser group had better visual acuity than either IVTA groups. In addition, the laser group had fewer incidences of cataract and glaucoma<sup>[27,58,59]</sup>.

In the second randomized controlled trial by the DRCR network, focal/grid laser alone was compared to 4 mg of intravitreal triamcinolone plus laser. Two additional arms utilized intravitreal ranibizumab. Similar to the previous study, the triamcinolone plus laser showed superiority compared to laser alone in terms of visual acuity at 24 wk follow-up. However, at one and two years, the treatments appeared equivalent in terms of visual acuity outcome, but with increased rates of cataract and elevated intraocular pressure in the triamcinolone plus laser group<sup>[60,61]</sup>. In the subgroup analysis of patients who were pseudophakic at baseline, the triamcinolone plus laser group appeared superior to the laser alone treatment and equivalent to the treatment arms utilizing ranibizumab<sup>[60,61]</sup>.

Recently, Gillies *et al*<sup>[62]</sup> reported the 24-mo results of a randomized controlled trial of intravitreal triamcinolone plus laser versus laser treatment only for diabetic macular edema which showed that treatment with IVTA plus macular laser resulted in a doubling of improvement in vision compared with laser only over 2 years in eyes with DME, but associated with cataract and raised intraocular pressure.

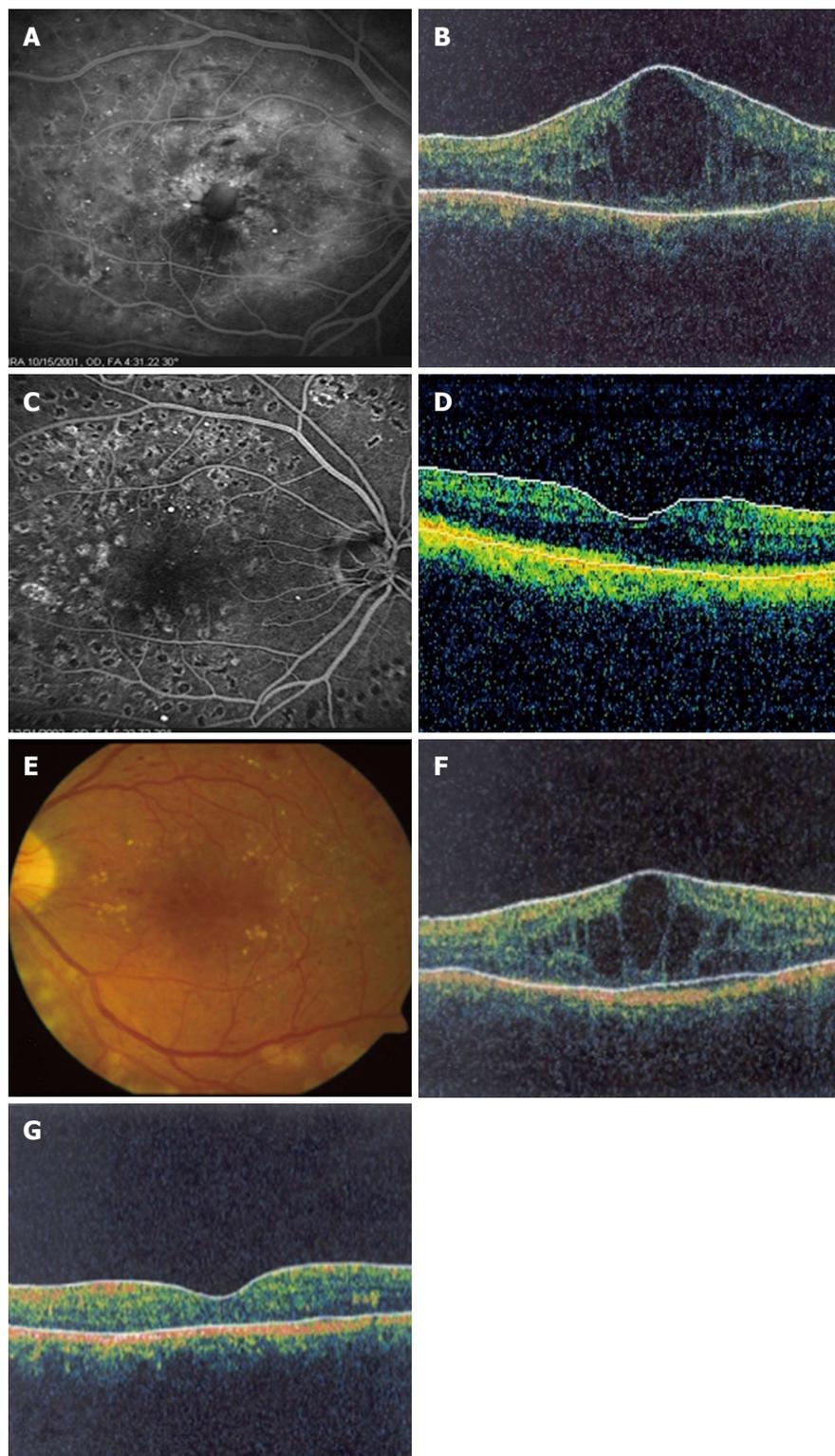


Figure 2 Diabetic macular edema before and after intravitreal triamcinolone acetonide injection.

The role of IVTA as adjunctive treatment to panretinal photocoagulation (PRP) for patients with proliferative diabetic retinopathy (PDR) and DME is being evaluated, and clinical studies have demonstrated the effectiveness of combination in preventing exacerbations of macular edema with improvement in visual acuity and macular

thickness in patients having PDR and DME<sup>[49-51,63,64]</sup>. However, a recent study demonstrated no beneficial effect of combined IVTA plus PRP and macular photocoagulation in coexisting high-risk PDR and DME in terms of visual acuity and macular thickness compared with standard treatment<sup>[65]</sup>.

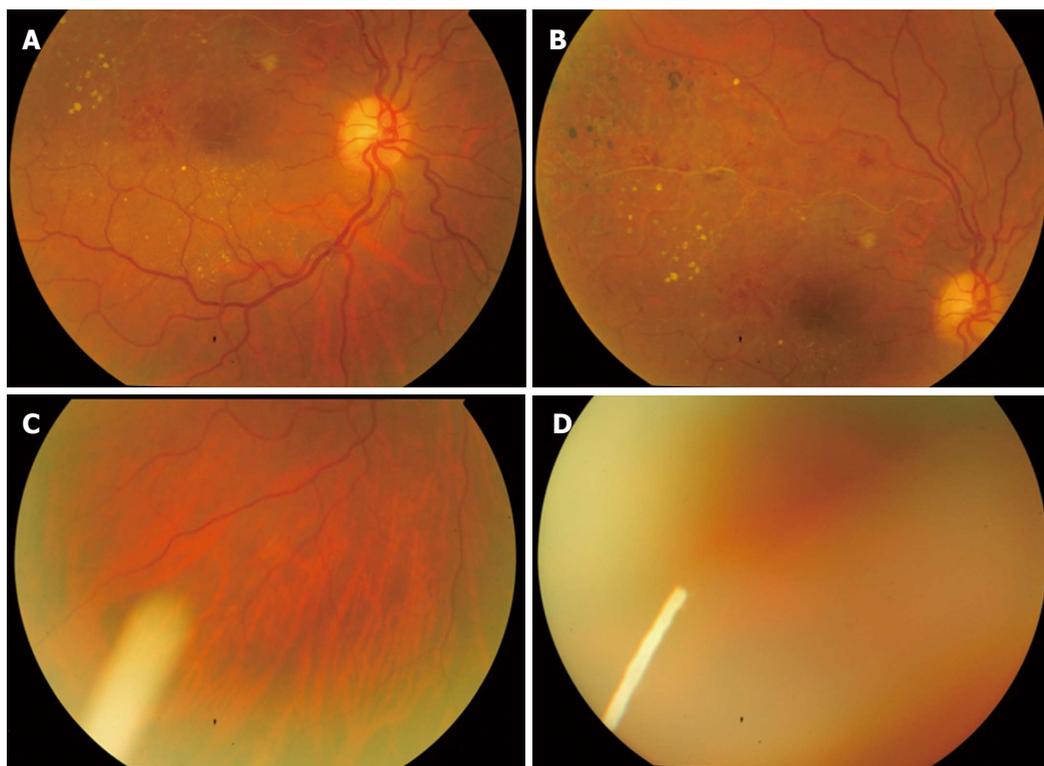


Figure 3 Ozurdex biodegradable dexamethasone injectable intravitreal implant in a patient with non-proliferative diabetic retinopathy, diabetic macular edema, and supero-temporal branch retinal vein occlusion.

### INTRAVITREAL STEROID SUSTAINED-RELEASE IMPLANTS

Nevertheless, the utilization of IVTA is not free of risks. The most common side-effects of IVTA are steroid-induced elevation of IOP varying from 20% to 70%, cataract in about 15%-20%, and crystalline maculopathy<sup>[18,19,21,22,52,66-71]</sup>. Other complications include endophthalmitis, intraocular hemorrhages, detachment of the retina<sup>[52,70,71-74]</sup>. In order to reduce the risks, complications and frequent dosing of intravitreal steroid, intravitreal steroid sustained-release implants have been developed.

Ozurdex (Allergan, Irvine, CA, United States) is a biodegradable dexamethasone injectable intravitreal implant approved by the FDA to treat patients with macular edema due to retinal vein occlusion, as well as noninfectious posterior uveitis (Figure 3). Studies have shown the benefit of Ozurdex in treating DME with improvement in visual acuity and macular thickness<sup>[75,76]</sup>. Analysis of 171 eyes with persistent DME that were treated with either 0.7 mg or 0.35 mg of Ozurdex, a best corrected visual acuity (BCVA) improvement of 10 letters or more was seen in more eyes in the 0.7 mg group (33.3%) and 0.35 mg group (21.1%) than the observation group (12.3%;  $P = 0.007$ ) with decreased central foveal thickness and leakage on fluorescein angiogram at 90 d compared to observation<sup>[75]</sup>. However, at 180 d, no significant difference was found between Ozurdex groups and the observation group. There was no significant difference in the number of patients with cataract among the study groups. Both

the 0.7-mg and 0.35-mg group had IOP elevation, 15% of patients who had received Ozurdex implants had an IOP increase of 10 mmHg or more from baseline, compared with 2% among patients from the observation group. All cases were successfully managed by observation or with topical IOP lowering medications. However, the rate of IOP elevation was lower than what has been reported for IVTA<sup>[75]</sup>. In addition, 0.7 mg of Ozurdex for treatment-resistant DME in vitrectomized eyes has been evaluated prospectively and showed significant improvement in both vision and vascular leakage from diabetic macular edema at 26 wk compared to baseline and may have a role in management of difficult to treat DME in vitrectomized eyes with acceptable safety profile<sup>[75,76]</sup>.

Retisert (Bausch and Lomb, Rochester, NY, United States) is another non-biodegradable sustained-release fluocinolone acetonide intravitreal implants that is approved by the FDA for the treatment of non-infectious uveitis. It is designed to release 0.59 mg/d of drug for about two-and-a-half years. Recently, the 3-year efficacy and safety results of a 4-year study evaluating fluocinolone acetonide (FA) intravitreal implants in eyes with persistent or recurrent DME has been published. They included 196 eyes with refractory DME. Patients were randomized 2:1 to receive 0.59-mg FA implant ( $n = 127$ ) or standard of care (SOC additional laser or observation;  $n = 69$ ). The implant was inserted through a pars plana incision. Overall, VA improved 3 lines in 16.8% of implanted eyes at 6 mo ( $P = 0.0012$ ; SOC, 1.4%); in 16.4% at 1 year ( $P = 0.1191$ ; SOC, 8.1%); in 31.8% at 2

years ( $P = 0.0016$ ; SOC, 9.3%); and in 31.1% at 3 years ( $P = 0.1566$ ; SOC, 20.0%). The number of implanted eyes with no evidence of retinal thickening at the center of the macula was higher than SOC eyes at 6 mo ( $P < 0.0001$ ), 1 year ( $P < 0.0001$ ; 72% *vs* 22%), 2 years ( $P = 0.016$ ), and 3 years ( $P = 0.861$ ). The most common adverse events included cataract progression in 91% of phakic eyes, and about 61% of implanted eyes had an IOP of  $\geq 30$  mmHg at any time and 33.8% required glaucoma surgery by 4 years. Despite cataract progression and elevated IOP, the 0.59-mg FA intravitreal implant significantly improved VA, Diabetic Retinopathy Severity Score, reduced central macular thickening, and may be used as an effective treatment for eyes with persistent or recurrent DME<sup>[77]</sup>.

Iluvien (Alimera Sciences, Alpharetta, GA, United States) is another FA implant, which is biodegradable and releases FA at a rate of either 0.2 or 0.5  $\mu\text{g}$  per day for up to three years. The Fluocinolone Acetonide for Macular Edema (FAME) studies conducted over a 36-mo period included a total of 956 patients with DME. The study consisted of two separate prospective, randomized, controlled, double-masked, multicenter clinical trials conducted to assess the efficacy and safety of low dose (releasing 0.2  $\mu\text{g}/\text{d}$ ) and high dose (0.5  $\mu\text{g}/\text{d}$ ) intravitreal FA (Iluvien) in patients with DME. At 24 mo, 28.7% of the low-dose and 28.6% of the high-dose group had 15 or more letter improvement, compared to 16.2% in the sham group ( $P = 0.002$  for each)<sup>[78]</sup>. At 24 mo, 3.7% of the low-dose, 7.6% of the high-dose, and 0.5% of the sham group required incisional glaucoma surgery. Cataract developed more frequently in the treatment group, with about 75% of the initially phakic patients undergoing cataract surgery at 24 mo<sup>[78]</sup>. At month 36, the percentage of patients who gained  $\geq 15$  in letter was 28.7% (low dose) and 27.8% (high dose) in the FA insert groups compared with 18.9% ( $P = 0.018$ ) in the sham group<sup>[79]</sup>. Preplanned subgroup analysis demonstrated a doubling of benefit compared with sham injections in patients who reported duration of DME  $\geq 3$  years at baseline; the percentage who gained  $\geq 15$  in letter score at month 36 was 34.0% (low dose;  $P < 0.001$ ) or 28.8% (high dose;  $P = 0.002$ ) compared with 13.4% (sham). At 36 mo, almost all phakic patients in the FA insert groups developed cataract, but their visual benefit after cataract surgery was similar to that in pseudophakic patients. The incidence of incisional glaucoma surgery at month 36 was 4.8% in the low-dose group and 8.1% in the high-dose insert group<sup>[80]</sup>.

Novel delivery approaches using sustained release approaches are likely to provide much needed benefit for patients with refractory DME resistant to more conventional and conservative therapy, though long-term data is so far not known. The need to perform initial surgery as well as the need to remove the empty device could be considered a hurdle to use non-biodegradable delivery systems releasing corticosteroids, also with many concerns about the side effects. Compared to other steroid

systems, the biodegradable dexamethasone delivery system is easier to apply and does not need to be removed. The safety and performance of an applicator-inserted dexamethasone drug delivery system was evaluated and also compared to the incisional placement. The results have revealed that the applicator-inserted dexamethasone drug delivery system performed well and effectively, and provided safe sutureless intravitreal placement of dexamethasone delivery system<sup>[79]</sup>.

## CONCLUSION

Despite the documented ocular side effects of corticosteroids, they are still considered as one of the effective adjunct modalities for the treatment of DME especially for refractory and persistent cases that failed to respond to standard conventional laser photocoagulation or anti-angiogenics. Corticosteroids have the advantage of repeatability, as long as the IOP and cataract side effects are not significant, due to their potent anti-inflammatory and anti-vascular endothelial growth factor effect that maintain and help in reducing blood retinal barrier disruption in diabetic patients. However recently, in order to reduce the risks, minimized the side effects and frequent dosing of intravitreal steroid injections, intravitreal steroid sustained-release implants have been developed.

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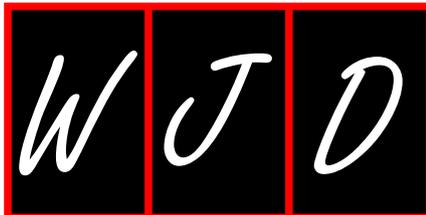
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## Vascular endothelial growth factor trap-eye (Aflibercept) for the management of diabetic macular edema

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### Abstract

Diabetic retinopathy (DR) is the most common cause of visual loss among working age individuals. Diabetic macular edema (DME) is an important complication of DR that affects around one third of the patients with DR. Several treatments have been approved for DME ranging from blood pressure and glycemic control to photocoagulation and more recently the use of vascular endothelial growth factor (VEGF) antagonists. The index review discusses aflibercept (EYLEA®-Regeneron Pharmaceuticals, Inc., Tarrytown, New York, NY, and Bayer Healthcare Pharmaceuticals, Berlin, Germany) in the context of other VEGF antagonists currently available for the treatment of DME. A systematic search of literature was conducted on PubMed, Scopus, and Google Scholar with no limitation on language or year of publication. Pre-clinical studies of aflibercept have shown a higher affinity of this molecule for vascular endothelial growth factor A (VEGF-A) along with a longer duration of action as compared to other VEGF antagonists. Recent clinical trials have shown visual

outcome results for aflibercept to be similarly favorable as compared to other available agents with the added benefit of fewer required injections and less frequent monitoring. Aflibercept presents a potential exciting new addition to the armamentarium of current VEGF antagonists available for the treatment of DME and other retinal vascular diseases. However, further studies are indicated to confirm the role, safety, and efficacy of aflibercept for DME.

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**Key words:** Diabetic macular edema; Diabetic retinopathy; Anti-vascular endothelial growth factor agents; Vascular endothelial growth factor trap-eye; Aflibercept; EYLEA

**Core tip:** Several different agents have been approved recently for the treatment of diabetic macular edema (DME). The index article outlines the role of aflibercept, an anti-vascular endothelial growth factor (VEGF) agent, as a potential therapeutic option. Results from DME trials with aflibercept have been favorable and comparable to other anti-VEGF agents. Because of its longer half-life, aflibercept may also decrease the frequency of injections for DME patients. These results could be attributed to the stronger and prolonged binding of aflibercept to the VEGF-A receptor compared to other available antagonists. A better understanding of the effect of this drug in DME is expected once the phase 3 trial results are available.

Moradi A, Sepah YJ, Sadiq MA, Nasir H, Kherani S, Sophie R, Do DV, Nguyen QD. Vascular endothelial growth factor trap-eye (Aflibercept) for the management of diabetic macular edema. *World J Diabetes* 2013; 4(6): 303-309 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/303.htm>  
DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.303>

## INTRODUCTION

In recent years, the rise in the incidence of diabetes mellitus (DM) has been accompanied by a significant increase in the incidence of its microvascular complications, including diabetic retinopathy<sup>[1]</sup>. Diabetic retinopathy (DR) is the most common cause of visual loss in working age individuals in developed nations<sup>[2,3]</sup>. Complications of diabetic retinopathy include: cataract, glaucoma, macular edema (ME), retinal hemorrhage and retinal detachment, among others.

Approximately one third of DR patients have ME while overall ME affects around 7.5% of diabetic population<sup>[4]</sup>.

Over the years the treatment of DR has ranged from tight glycemic control to partial pituitary destruction and it was not until the Early Treatment Diabetic Retinopathy Study (ETDRS) showed efficacy of laser in treating DR/DME (Diabetic macular edema) that this modality was recognized as a useful intervention. It was demonstrated that laser photocoagulation can reduce the risk of moderate visual loss by 50% three years after initiation of treatment and was thus approved for the treatment of DME<sup>[5]</sup>. However, this form of treatment often prevents further visual loss and rarely results in improvement of vision. Therefore, researchers have explored newer therapies to improve treatment outcomes for patients with DME<sup>[6,7]</sup>.

## RATIONALE FOR CONSIDERING VEGF ANTAGONISTS FOR DME

DME results from microvascular changes secondary to hyperglycemia and the inflammatory changes associated with diabetes. These include thickening of the basement membrane and loss of pericytes, which leads to increased permeability of vessels leading to edema formation. Associated hypoxia due to vascular occlusion also leads to the release of several cytokines such as vascular endothelial growth factor (VEGF), insulin-like growth factor-1, angiopoietin-1 and angiopoietin-2, stromal-derived factor-1, fibroblast growth factor-2, and tumor necrosis factor<sup>[8]</sup>.

In 1994 Aiello *et al.*<sup>[9]</sup> demonstrated that VEGF levels are elevated in the ocular fluid of patients with diabetic retinopathy and other retinal disorders, including DME. Subsequently, Ozaki *et al.*<sup>[10]</sup> and Tolentino *et al.*<sup>[11,12]</sup> showed that intravitreal injection of VEGF in nonhuman primates could generate similar findings of diabetic retinopathy such as micro aneurysms, macular edema, and retinal neovascularization. Recent studies have also confirmed that hypoxia-induced VEGF release has a key role in the pathophysiology of DME<sup>[13]</sup>. During the past decade, the scientific community has learned that VEGF leads to neovascularization, increased vascular permeability and breakdown of the blood retina barrier, leading to the formation of ME<sup>[14]</sup>.

The establishment of the VEGF pathway leading to

ME in patients with diabetes and age-related macular degeneration (AMD) led to the development of anti VEGF therapies that are briefly discussed below.

## VEGF ANTAGONISTS THAT HAVE BEEN EVALUATED FOR DME

Pegaptanib sodium (Macugen, Eyetech Pharmaceuticals, Melville, NY/Pfizer, New York, NY) is an anti-VEGF aptamer that blocks the effect of VEGF by binding to the VEGF-A<sub>165</sub> isoform. It was the first United States Food and Drug Authority (FDA) approved anti-VEGF agent for treatment of choroidal neovascularization (CNV) resulting from AMD<sup>[15,16]</sup>.

Cunningham *et al.*<sup>[17]</sup> demonstrated in a double-masked phase 2 trial that subjects with DME who were assigned to the pegaptanib arm had better visual acuity (VA) outcomes [a larger proportion of those receiving 0.3 mg gained VAs of  $\geq 10$  letters as compared to the sham group (34% *vs* 10%,  $P = 0.003$ ) and  $\geq 15$  letters (18% *vs* 7%,  $P = 0.12$ )], were more likely to show reduction in central retinal thickness [Mean central retinal thickness decreased by 68  $\mu\text{m}$  with 0.3 mg, *vs* an increase of 4  $\mu\text{m}$  with sham ( $P = 0.02$ )], and were deemed less likely to need additional therapy with photocoagulation at follow-up as compared to sham injections at week 36. Phase 3 trials are currently underway<sup>[18]</sup>.

Ranibizumab (Lucentis<sup>TM</sup>, Genentech, San Francisco, CA) is a humanized antibody fragment that binds to all isoforms of VEGF-A. In 2006, Nguyen and colleagues were among the first clinician-scientists in the world to demonstrate that VEGF plays a critical role in the pathogenesis of DME and that employment of a VEGF antagonist such as ranibizumab may help to reduce retinal edema<sup>[19]</sup>. The READ-2 study, a phase II, randomized clinical trial, was conducted to evaluate the efficacy of ranibizumab for DME. Subjects were randomized 1:1:1 to receive 0.5 mg ranibizumab (group 1), focal or grid laser photocoagulation (group 2), or a combination of laser and ranibizumab (group 3). After the primary end point at month 6, at the discretion of the investigators, the majority of patients were treated only with ranibizumab. The mean improvement in BCVA was 7.24, 0.43, and 3.8 letters at the 6 mo primary end point<sup>[20]</sup>, compared with 7.7, 5.1, and 6.8 letters at month 24. Mean foveal thickness (FTH), defined as center subfield thickness, at month 24 was 340  $\mu\text{m}$ , 286  $\mu\text{m}$ , and 258  $\mu\text{m}$  for groups 1, 2, and 3, respectively<sup>[21]</sup>.

The RISE and RIDE studies, two randomized, multicenter and double masked phase 3 trials, have shown that ranibizumab improved vision and macular edema in patients with DME and reduced the risk of further visual loss. The results of the RISE study showed that 18.1% of sham patients gained  $\geq 15$  letters *vs* 44.8% of 0.3-mg ( $P < 0.0001$ ) and 39.2% of 0.5-mg ranibizumab patients ( $P < 0.001$ ). In RIDE, 12.3% of sham patients gained  $\geq 15$  letters *vs* 33.6% of 0.3-mg patients ( $P < 0.0001$ ) and 45.7%

of 0.5-mg ranibizumab patients ( $P < 0.0001$ ). Based on such significant results from RISE and RIDE, the FDA approved ranibizumab for DME in August 2012<sup>[22]</sup>.

Bevacizumab (Avastin®, Genentech, San Francisco, CA) is a full-length recombinant humanized antibody that targets all isoforms of VEGF-A. It is approved by the FDA as adjunctive treatment for metastatic colonic cancer but has not yet been approved for ocular diseases. The intravitreal Bevacizumab or Laser Therapy in the Management of Diabetic Macular Edema (BOLT) study, which was conducted on patients with center-involving clinically significant macular edema (CSME), showed a mean gain of 8.6 letters in the bevacizumab group as compared to a mean loss of 0.5 letters for the macular laser therapy group<sup>[23]</sup>.

Table 1 outlines a summary of landmark clinical trials in which VEGF antagonists were evaluated for DME.

## INTRODUCTION TO VEGF TRAP EYE/ AFLIBERCEPT

VEGF Trap-Eye/Aflibercept (EYLEA®-Regeneron Pharmaceuticals, Inc., Tarrytown, New York, NY, and Bayer Healthcare Pharmaceuticals, Berlin, Germany) is a novel 115-kDa anti-VEGF agent. This fusion protein has been developed using the “trap technology” in which the extracellular binding domains of VEGF receptor (VEGFR) -1 and -2 are combined to the Fc segment of human immunoglobulin-G1 backbone<sup>[24]</sup>. Similar to ranibizumab and bevacizumab, aflibercept binds to all isomers of the VEGF-A family. Additionally, aflibercept binds to VEGF-B and placental growth factor; it is hypothesized that by blocking these factors, aflibercept may prove to be more efficacious<sup>[25]</sup>.

As a soluble circulation trap for VEGF, aflibercept binds firmly to the target, clears it out from the vitreous<sup>[26]</sup>, and consequently inhibits binding and activation of the VEGF receptors. The affinity of aflibercept to VEGF-A is much higher than monoclonal anti-VEGF antibodies (5 pM *K<sub>d</sub>* vs 1 pM *K<sub>d</sub>*).

The receptor sequences of aflibercept provide powerful VEGF binding (140 times that of ranibizumab) and the molecule's intermediate size of 110 kD (compared to 48 kD for ranibizumab and 148 kD for bevacizumab) creates a one-month intravitreal binding activity that exceeds both ranibizumab and bevacizumab<sup>[27]</sup>.

According to Stewart *et al.*<sup>[27]</sup>, a predictive model showed that aflibercept is active in the eye for 10 to 12 wk after a single intravitreal injection, with the binding activity of 2 mg VEGF Trap-Eye at 83 d estimated to be comparable to that of 0.5 mg ranibizumab at 30 d.

The half-life of intravitreally-administered aflibercept in animals is about 5 d; however, the half-life in the human eye has not yet been identified. Based on a mathematical model, this half-life is estimated to be about 7.13 d<sup>[28,29]</sup>.

### Dosage and safety

Aflibercept is available in a single-use vial which contains

0.05 mL of aflibercept (40 mg/mL in 10 mmol/L sodium phosphate, 40 mmol/L sodium chloride, 0.03% polysorbate 20, and 5% sucrose, pH 6.2)<sup>[30]</sup>. To date, aflibercept is only available for intravitreal (IVT) injection. Although intravenous administration of doses  $\geq 1$  mg/kg has caused some systemic adverse events, no systemic effects have been reported in any phase-I, phase-II, or phase-III trials with IVT administration of doses of up to 4 mg ( $< 0.06$  mg/kg; 0.057 mg/kg)<sup>[31-33]</sup>.

## AFLIBERCEPT FOR DME: CURRENT EVIDENCE

In 2009, a phase-I study was conducted by Do *et al.*<sup>[31]</sup> to assess the safety, tolerability and bioactivity of a single 4 mg intravitreal injection of aflibercept in five patients with DME over a period of 6 wk. Participants older than 18 years with type 1 or type 2 diabetes, retinal thickening involving the foveal center due to DME, foveal thickness  $\geq 250$   $\mu$ m as measured by Stratus OCT, and BCVA between 20/40 and 20/320, were included in this study. The biological activity was measured by alterations in BCVA as well as changes in the retinal thickness assessed by OCT. The results of this trial established that a single intravitreal injection of 4 mg of aflibercept was well tolerated without any ocular toxicity. Its biologic activity was recognized by its role in improving BCVA and reducing retinal thickness. The median BCVA was 36 letters at baseline, and the median improvement seen was 9 letters after 4 wk and 3 letters after 6 wk. The median excess central 1mm foveal thickness (FTH) was 108  $\mu$ m at baseline, which was reduced to 59  $\mu$ m at 4 wk and 74  $\mu$ m at 6 wk after injection. Four of the five patients maintained the improvement in excess FTH (median 74 mm; 31% reduction from baseline,  $P = 0.0625$ ) at 6 wk after injection.

Based on the results of the phase I study, a 52 wk, multicenter, randomized, double-masked, active-controlled phase II clinical trial was conducted. The primary aim of the DME and VEGF Trap-Eye: INvestigation of Clinical Impact (DA VINCI) Study was to assess the safety and efficacy of intravitreal aflibercept in comparison with focal/grid laser photocoagulation in patients with DME. Diabetic patients who were  $\geq 18$  years old, with central retinal thickness (CRT)  $\geq 250$   $\mu$ m in the central subfield based on Stratus® OCT and with a BCVA between 20/40 and 20/320 were included in this study. Important study exclusion criteria were: history of vitreoretinal surgery, panretinal or macular laser photocoagulation within 3 mo of screening, use of intraocular or periocular corticosteroids within 3 mo of screening and uncontrolled diabetes mellitus or hypertension.

In the DA VINCI study, a total of 221 patients with DME from 39 sites in the United States, Canada, and Austria were randomly assigned in a 1:1:1:1:1 ratio to one of 5 treatment regimens in one eye only: 0.5 mg aflibercept every 4 wk (0.5q4); 2 mg aflibercept every 4 wk (2q4); 2 mg aflibercept for 3 initial monthly doses and then every 8 wk (2q8); 2 mg aflibercept for 3 initial monthly

**Table 1 Summary of some important trials in which vascular endothelial growth factor antagonists have been evaluated for diabetic macular edema**

Trial	Drug name						
	Pegaptanib	Bevacizumab	RESOLVE <sup>[40]</sup>	READ-2 <sup>[21,20]</sup>	READ-3	Ranibizumab	RIDE <sup>[22]</sup>
Study name	Study to Evaluate safety and tolerability of pegaptanib sodium in patients with diabetic macular edema <sup>[39]</sup>	BOLT study <sup>[23,39]</sup>	RESOLVE <sup>[40]</sup>	READ-2 <sup>[21,20]</sup>	READ-3	RISE <sup>[22]</sup>	RIDE <sup>[22]</sup>
Study type/phase	Interventional /phase 3	Interventional /phase 2	Interventional /phase 2	Interventional /phase 2	Interventional /phase 2	Interventional /phase 3	Interventional /phase 3
Number of patients	46	80	151	126	142	377	382
Intervention/study design	0.3 mg injections up to a maximum of 48 wk with a minimum-dosing interval of at least 6 wk	(1) Intravitreal Bevacizumab (2) MLT	(1) 0.3 mg Ranibizumab (2) 0.5 mg Ranibizumab (3) Sham	(1) 0.5 mg ranibizumab (group 1) (2) Focal/ grid laser (group 2) (3) 0.5 mg ranibizumab + focal/ grid laser (group 3)	(1) 0.5 mg ranibizumab (2) 2 mg ranibizumab	(1) Sham (2) 0.3 mg (3) 0.5 mg	(1) Sham (2) 0.3 mg (3) 0.5 mg
Results	No results available yet	Year 1 endpoint: A median gain of 8 ETDRS letters in the bevacizumab group <i>vs</i> a loss of 0.5 ETDRS letters in the MLT group ( $P = 0.0002$ ) CMT decreased from $507 \pm 145 \mu\text{m}$ to $378 \pm 134 \mu\text{m}$ ( $P < 0.001$ ) in the bevacizumab group, whereas it decreased from $481 \pm 121 \mu\text{m}$ to $413 \pm 135 \mu\text{m}$ in the MLT group ( $P = 0.02$ ) <sup>[39]</sup> Year 2 endpoint: A Mean gain of 8.6 letters for bevacizumab <i>vs</i> a mean loss of 0.5 letters in the MLT group <sup>[23]</sup> . A mean reduction of 146 $\mu\text{m}$ in the CMT in the bevacizumab arm <i>vs</i> 118 $\mu\text{m}$ in the MLT arm	A gain of $10.3 \pm 9.1$ letters with a loss of $1.4 \pm 14.2$ letters in the sham group ( $P < 0.0001$ ) A mean CMT reduction of $194.2 \pm 135.1 \mu\text{m}$ with ranibizumab and $48.4 \pm 153.4 \mu\text{m}$ with sham ( $P < 0.0001$ ) A gain of $\geq 10$ letters in BCVA from baseline in 60.8% of eyes in the ranibizumab group and 18.4% of eyes in the sham group ( $P < 0.0001$ )	The mean improvement in BCVA was 7.4, 0.5, and 3.8 letters at the 6 mo primary end point, compared with 7.7, 5.1, and 6.8 letters at month 24 in group 1, group 2 and group 3 respectively The percentage of patients who gained 3 lines or more of BCVA was 21, 0, and 6 at month 6, compared with 24, 18, and 26 at month 24. Mean FTH, defined as center subfield thickness, at month 24 was 340 $\mu\text{m}$ , 286 $\mu\text{m}$ , and 258 $\mu\text{m}$ for groups 1, 2, and 3, respectively	The study has completed Results are being analyzed	Year 2 endpoint: $\geq 15$ letters <i>vs</i> 44.8% of 0.3-mg ( $P < 0.0001$ ) and 39.2% of 0.5-mg ranibizumab patients ( $P < 0.001$ )	12.3% of sham patients <i>vs</i> 33.6% of 0.3-mg patients ( $P < 0.0001$ ) and 45.7% of 0.5-mg ranibizumab patients ( $P < 0.0001$ ) gained more than 15 letters

DME: Diabetic macular edema; MLT: Macular laser therapy; FTH: Foveal thickness; BOLT: The intravitreal Bevacizumab or Laser Therapy in the Management of Diabetic Macular Edema; READ: The Ranibizumab for Edema of the mAcula in diabetes; CMT: Central macular thickness.

doses and then on an as-needed (PRN) basis (2PRN); or macular laser photocoagulation as specified by the modified ETDRS protocol. Assessments were done at baseline and every 4 wk thereafter.

The primary end point results of the DA VINCI

study (week 24) revealed that treatment with intravitreal aflibercept produced a statistically significant improvement in VA when compared with macular laser treatment. The four aflibercept groups showed a greater mean BCVA gain and decrease in CRT in comparison to the

**Table 2** Summary of clinical trials in which aflibercept was evaluated for diabetic macular edema

Trial	Study Name					
	Phase 1 study of VEGF trap in patients with DME	DA VINCI	VIVID-Japan	VISTA DME	VIVID-DME	Protocol T
Study type	Interventional	Interventional	Interventional	Interventional	Interventional	Interventional
Study phase	Phase 1	Phase 2	Phase 3	Phase 3	Phase 3	Phase 3
Official title	An exploratory study of the safety, tolerability and biological effect of a single intravitreal administration of VEGF trap eye in patients with DME	A double-masked, randomized, controlled study of the safety, tolerability and biological effect of repeated intravitreal administration of VEGF trap-eye in patients with DME	A randomized, double masked, active controlled, phase III study of the efficacy and safety of repeated doses of intravitreal VEGF trap-eye in subjects with DME	A double-masked, randomized, active-controlled, phase 3 study of the efficacy and safety of intravitreal administration of VEGF trap-eye in patients with DME	An open-label phase III study evaluating the safety and tolerability of repeated doses of intravitreal VEGF trap-eye in Japanese subjects with DME	A comparative effectiveness study of intravitreal aflibercept, bevacizumab and ranibizumab for DME
Study design						
Allocation	Non-randomized	Randomized	Randomized	N/A	Randomized	Randomized
Endpoint classification	Safety Study	Safety/Efficacy study	Safety/Efficacy study	Safety/Efficacy study	Safety/Efficacy study	Safety/Efficacy study
Intervention model	Single group assignment	Parallel assignment	Parallel assignment	Single group assignment	Parallel assignment	Parallel assignment
Masking	Open label	Double blind (subject, investigator, outcomes assessor)	Double blind (subject, investigator, outcomes assessor)	Open label	Double blind (subject, investigator, outcomes assessor)	Single blind (subject)
Primary purpose	Treatment	Treatment	Treatment	Treatment	Treatment	Treatment
Enrollment	5	219	65	466	406	660
Study period	6 wk	52 wk	48 wk	2 yr	52 wk	2 yr
Recruitment status	Completed	Completed	Recruiting	Active, not recruiting	Active, not recruiting	Active, recruiting
Primary outcome measure	To assess the ocular and systemic safety and tolerability of a single IVT injection of VEGF Trap-eye in patients with DME	Change in BCVA	Adverse event collection	Change from baseline of BCVA in ETDRS letter score	Change from baseline of BCVA in ETDRS letter score	Change in visual acuity single blind adjusted for baseline visual acuity
Estimated study completion date	Completed	Completed	2013	2014	2015	2016

BCVA: Best corrected visual acuity; DME: Diabetic macular edema; VEGF: Vascular endothelial growth factor; ETDRS: Early treatment diabetic retinopathy study.

laser group. [+8.5 to +11.4 ETDRS letters *vs* only +2.5 letters in the laser group ( $P \leq 0.0085$  for each aflibercept group *vs* laser) and +127.3 to +194.5  $\mu\text{m}$  *vs* +67.9  $\mu\text{m}$  ( $P = 0.0066$  for each aflibercept group *vs* laser)]. It also showed that aflibercept was well tolerated and its ocular adverse events were consistent with those seen with other intravitreal anti-VEGF agents.

Recently, the DA VINCI study group has also published the results of different doses and dosing regimens of aflibercept with laser photocoagulation in eyes with DME after 52 wk. Assessment of the changes in BCVA and mean changes in CRT at 24 and 52 wk revealed that significant gains in BCVA from baseline, achieved at week 24, were maintained or improved at week 52 in all aflibercept groups. Mean BCVA increases in the aflibercept groups at week 52 were 11.0, 13.1, 9.7, and 12.0 letters *vs* -1.3 letters for the laser group ( $P \leq 0.0001$ ).

Mean decrease in CRT in the aflibercept groups at week 52 were -165.4, -227.4, -187.8, and -180.3  $\mu\text{m}$  *vs* -58.4  $\mu\text{m}$  for laser ( $P < 0.0001$ )<sup>[34]</sup>.

At one year, subjects who were treated with aflibercept in the DA VINCI study were doing well. The most frequently reported ocular adverse events associated with aflibercept use were conjunctival hemorrhage (18.9%), increased intraocular pressure (9.7%), eye pain (8.6%), ocular hyperemia (6.3%) and vitreous floaters (5.1%). Serious adverse effects included endophthalmitis (1.1%), uveitis (0.6%), corneal abrasion (0.6%) and retinal tear (0.6%). Systemic adverse events included hypertension (9.7%), cerebral vascular accidents (1.1%), and myocardial infarction (1.1%).

## ONGOING STUDIES

Since the DA VINCI study was not powered sufficiently

to uncover the potential systemic AEs or mortality related to aflibercept, additional phase-III clinical studies of aflibercept have been initiated (Table 2).

### VIVID-Japan

The Japanese Safety Study of VEGF Trap-Eye in DME (VIVID-Japan) is an open-label phase-III study evaluating the safety and tolerability of repeated doses of intravitreal VEGF Trap-Eye (BAY86-5321) in Japanese subjects with DME. Subjects in the aflibercept group will initially receive a loading phase consisting of injections given every 4 wk, followed by an injection every 8 wk. The overall treatment period is 48 wk<sup>[35]</sup>.

### VIVID-DME

VEGF Trap-Eye in Vision Impairment Due to DME (VIVID-DME) was designed to determine the efficacy of intravitreal VEGF Trap-Eye (BAY86-5321) on the BCVA assessed by the ETDRS chart in subjects with DME with central involvement. This multi-central, randomized, double masked, active controlled, phase-III study of the efficacy and safety of repeated doses of intravitreal aflibercept in subjects with DME will assess changes from baseline of BCVA in ETDRS letter score over a 52 wk period in about 375 patients in 91 study locations. The treatment regimens will include 2 arms treated with aflibercept and one with laser therapy according to the ETDRS protocol<sup>[36]</sup>.

### VISTA DME

Study of Intravitreal Administration of VEGF Trap-Eye (BAY86-5321) in Patients with Diabetic Macular Edema (VISTA DME) will assess the efficacy of 2 different dosing regimens of aflibercept compared with laser over the course of 2-year period. VISTA DME is a double-masked, randomized, active-controlled, phase-III study of the efficacy and safety of intravitreal aflibercept in 466 subjects with DME in 52 study locations<sup>[37]</sup>.

### Protocol T

This phase III study sponsored by the Diabetic Retinopathy Clinical Research Network will compare the safety and efficacy of intravitreal aflibercept (2.0 mg), bevacizumab (1.25 mg) and ranibizumab (0.5 mg) for DME in 660 patients recruited from different clinical centers in the US. The primary outcome in this study is to evaluate the changes in BCVA at month 12. Protocol T is currently recruiting patients<sup>[38]</sup>.

## ACKNOWLEDGMENTS

Dr. Do and Dr. Nguyen serve on the Steering Committee for the study of VEGF Trap Eye/Aflibercept in Diabetic Macular Edema. The Stanley M. Truhlsen Eye Institute has received funding support from Research to Prevent Blindness.

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## Ranibizumab in diabetic macular edema

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### Abstract

By 2050 the prevalence of diabetes will more than triple globally, dramatically increasing the societal and financial burden of this disease worldwide. As a consequence of this growth, it is anticipated that there will be a concurrent rise in the numbers of patients with diabetic macular edema (DME), already among the most common causes of severe vision loss worldwide. Recent available therapies for DME target the secreted cytokine, vascular endothelial growth factor (VEGF). This review focuses on the treatment of DME using the first humanized monoclonal antibody targeting VEGF that has been Food and Drug Administration-approved for the use in the eye, ranibizumab (Lucentis®).

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**Key words:** Diabetes; Macular edema; Vascular endothelial growth factor; Ranibizumab; Lucentis®; Bevacizumab; Avastin®; Intravitreal injection

**Core tip:** This article reviews the use of ranibizumab for

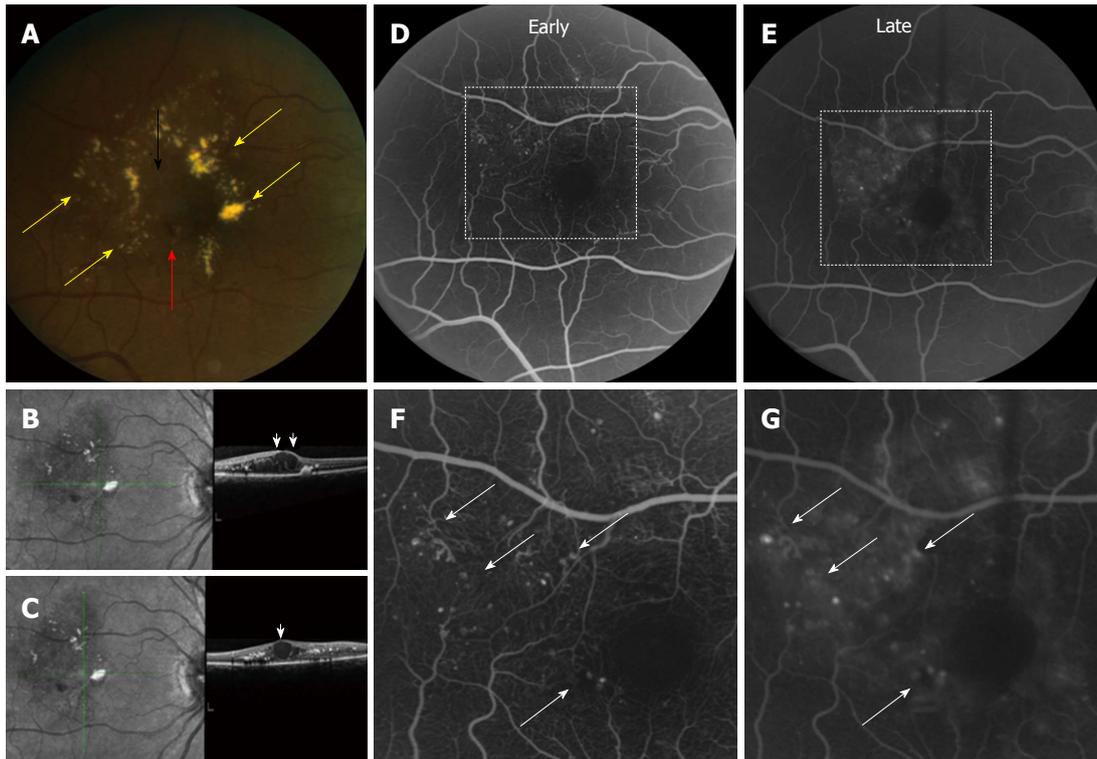
diabetic macular edema. The article presents recent data on which the practice of ranibizumab injections for diabetic macular edema is based, and highlights issues regarding efficacy, safety, and other important considerations for any retina provider using ranibizumab in practice.

Krispel C, Rodrigues M, Xin X, Sodhi A. Ranibizumab in diabetic macular edema. *World J Diabetes* 2013; 4(6): 310-318 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/310.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.310>

### INTRODUCTION

Diabetic retinopathy is a leading cause of visual impairment worldwide, and diabetic macular edema (DME) is the most common cause of vision loss in these patients<sup>[1]</sup>. DME is a consequence of breakdown of the vascular inner blood-retinal barrier (BRB), and may occur in one of two ways<sup>[2]</sup>: (1) focal leakage arising from microaneurysms; and (2) diffuse leakage arising from the walls of capillaries (Figure 1). Systemic disease plays a role in the development and progression of diabetic retinopathy, and improving control of blood glucose and blood pressure has been shown to slow the progression of diabetic eye disease<sup>[3]</sup>. However, in many patients, despite adequate diabetic control, if left untreated DME can result in significant vision loss<sup>[4]</sup>.

Since 1985, the mainstay of treatment for clinically significant (diabetic) macular edema had been focal/grid laser photocoagulation based on the Early Treatment Diabetic Retinopathy Study (ETDRS)<sup>[4]</sup>. In focal/grid laser photocoagulation, focal laser is applied to leaking microaneurysms while a grid pattern of larger burns of light intensity at the level of the retinal pigment epithelium is used to treat diffuse leakage. In the ETDRS, the 754 eyes treated with laser photocoagulation had approximately a 50% decrease in the risk of vision loss (defined as doubling of the initial visual angle or a loss of three or more



**Figure 1** Fundus photo, optical coherence tomography, and fluorescein angiography of a patient with diabetic macular edema. A: Fundus photo demonstrating classic presentation of diabetic macular edema with lipid exudate (yellow arrows), retinal thickening (black arrow), and intraretinal hemorrhages (red arrow); B, C: Horizontal (above) and vertical (below) high-resolution line scan demonstrating the presence of intraretinal cysts (white arrowheads) in the inner retina; D, E: Fluorescein angiographic images demonstrating focal leakage arising from microaneurysms (white arrows); F, G: Diffuse leakage arising from the walls of capillaries.

lines) compared to the 1490 eyes without treatment. However, laser photocoagulation did not significantly improve vision in patients enrolled in the ETDRS.

In 2002, a National Eye Institute-sponsored collaborative network, the Diabetic Retinopathy Clinical Research Network (DRCR.net), compared steroid versus laser treatment for DME<sup>[5]</sup>. In this study, 26% of DME patients treated with laser did gain 15 or more letters of vision at three years. Nonetheless, despite these promising results, laser photocoagulation for DME leaves the majority of patients with little hope for an improvement in vision. Thus, while many ophthalmologists continue to employ this modality of treatment, other approaches (*e.g.*, intravitreal steroids) have also been explored (alone or in combination with laser photocoagulation) with the goal of improving on these results; the use of intravitreal steroids for the treatment of DME is discussed elsewhere in this review series.

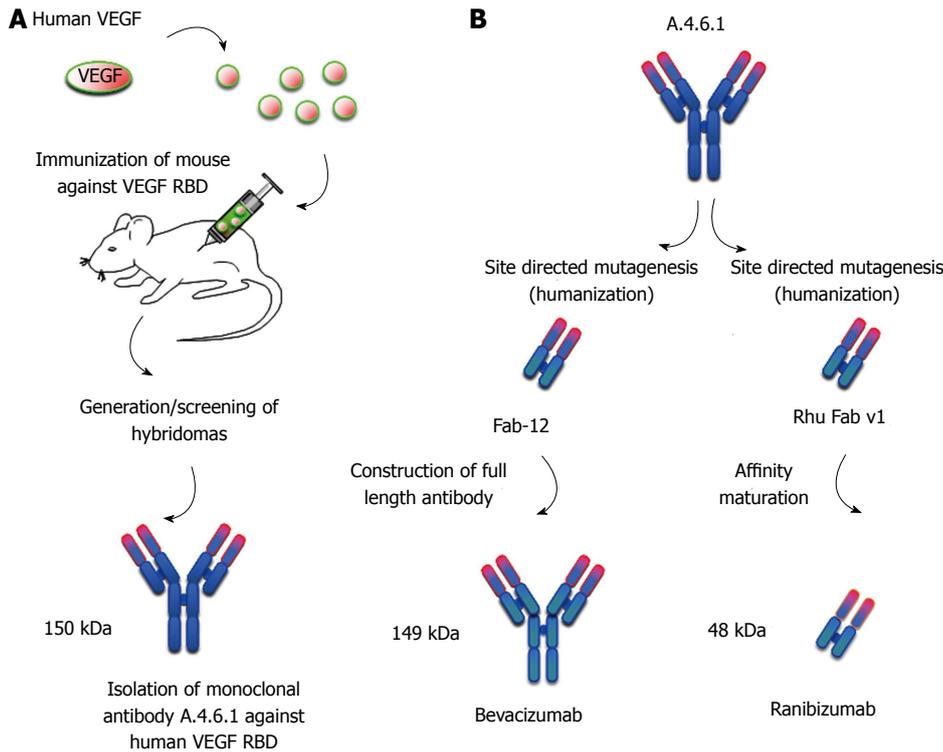
## VASCULAR ENDOTHELIAL GROWTH FACTOR

In this regard, recent efforts to unravel the molecular pathogenesis of DME have led to the development of new therapeutic approaches for the treatment of this disease. The concept that ischemic retinopathies are driven by a secreted angiogenic factor was proposed over half a century ago<sup>[6]</sup>. More recently, appreciation for a single cy-

tokine, vascular endothelial growth factor (VEGF), as the central player in the development of DME has facilitated a paradigm shift in how we treat this disease<sup>[7]</sup>.

VEGF is a sub-family of growth factors (the platelet-derived growth factor family of cysteine-knot growth factors) produced by hypoxic cells that act as signal proteins to stimulate both vasculogenesis and angiogenesis<sup>[8]</sup>. The VEGF sub-family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). These proteins act by binding to VEGF receptors (*e.g.*, VEGFRs 1-3), which are tyrosine kinase receptors with an extracellular ligand binding domain and an intracellular tyrosine kinase domain<sup>[9]</sup>. Upon ligand (VEGF) binding to the receptor, the receptors dimerize, and the tyrosine kinase domain initiates phosphorylation at the C-terminus of the molecule. This initiates an intracellular signaling cascade that ultimately leads to changes in gene transcription<sup>[9]</sup>.

VEGF affects a number of cell types (*e.g.*, monocytes and macrophages, neurons, tumor cells, kidney epithelial cells). However, VEGFR2 is expressed predominantly on vascular endothelial cells, which are predominantly responsible for the pathological effects of VEGF in the eye. In particular, VEGF-A has been shown to promote the growth and survival of vascular endothelial cells (promoting angiogenesis) and to disrupt endothelial cell-endothelial cell tight junctions (promoting vascular permeability), leading to retinal neovascularization and macular edema in diabetic eye disease, respectively<sup>[7]</sup>.



**Figure 2** Development of humanized neutralizing monoclonal antibodies against vascular endothelial growth factor. A: A polypeptide from the vascular endothelial growth factor (VEGF) receptor-binding domain (RBD) was used to immunize mice. The spleen from the mice was isolated and lymphocytes were cultured with immortal myeloma cell lines. Fusion of the cell lines results in the generation of hybridomas. Supernatants from the hybridoma cultures are tested for antibodies that bind specifically and with high affinity to VEGF. This resulted in the identification of the parental mouse monoclonal antibody, A.4.6.1; B: Site directed mutagenesis was then used to “humanize” the Fab (epitope-binding) fragment of A.4.6.1. Fab-12 was used to construct the full-length antibody, bevacizumab (left). Rhu Fab v1 was further affinity matured to produce the Fab fragment, ranibizumab (right).

## ANTI-VEGF THERAPIES

The rationale for using anti-VEGF agents to treat diseases characterized by dysregulated angiogenesis is based largely on studies demonstrating the role of VEGF in the context of cancer, where this angiogenic cytokine has been shown to play a critical role in tumor growth and metastasis<sup>[10]</sup>. A role for VEGF has subsequently been established in pathological angiogenesis in other diseases, including those affecting the eye<sup>[11]</sup>. Indeed, increased levels of VEGF have been demonstrated in the eyes of patients with diabetic retinopathy and diabetic macular edema compared to normal controls<sup>[12,13]</sup>. Collectively, these observations have prompted exploration of therapies targeting VEGF as an approach for patients with DME.

The first attempt to inhibit VEGF in ocular disease was with the pegylated anti-VEGF aptamer, Pegaptanib (Macugen<sup>®</sup>), a single strand of nucleic acid that binds with specificity to VEGF-165 mRNA<sup>[14]</sup>. Pegaptanib was originally developed and approved by the United States Food and Drug Administration (FDA) in December 2004 as an anti-angiogenic medicine for the treatment of neovascular (wet) age-related macular degeneration (AMD). The use of Pegaptanib for DME was also explored, and although it was not as effective as investigators had hoped, it did confirm the therapeutic potential of anti-VEGF therapy for diabetic eye disease<sup>[15]</sup>.

An alternative approach developed by Genentech to target VEGF was the use of humanized monoclonal antibodies targeting VEGF-A. Since VEGF is a secreted protein, it was vulnerable to targeting in the extracellular environment. The first monoclonal antibody against VEGF developed by Genentech was bevacizumab (Avastin<sup>®</sup>). Bevacizumab is a recombinant humanized monoclonal immunoglobulin G1 $\kappa$  antibody that was FDA-approved in February 2004 and marketed as a treatment for colon cancer<sup>[16]</sup>. However, as emerging evidence pointed to VEGF as a central player in ocular disease, ophthalmologists began to use bevacizumab as an “off-label” treatment for wet AMD and later diabetic eye disease<sup>[17,18]</sup>; the use of intravitreal bevacizumab for the treatment of DME is discussed briefly in this review and extensively elsewhere in this review series.

Soon after the release of bevacizumab, Genentech developed a second humanized monoclonal antibody targeting VEGF-A, ranibizumab (Lucentis<sup>®</sup>) that was FDA-approved in June 2006 and marketed for use in the eye<sup>[19]</sup>. Ranibizumab was created from the same parent mouse anti-human VEGF monoclonal antibody (mAb A.4.6.1) as was bevacizumab, and targets the identical epitope of VEGF (AS82-91) from within the receptor-binding domain (AS8-109) of VEGF165 (Figure 2). Ranibizumab underwent affinity purification to improve its binding to VEGF. And like bevacizumab, ranibizumab has neutralizing activity on all VEGF isoforms. However,

there are five important differences between bevacizumab and ranibizumab: (1) ranibizumab (48 kDa) contains only the Fab fragment of the parental antibody, while bevacizumab (149 kDa) contains the Fab and Fc fragments (whole antibody); (2) the sequence of ranibizumab differs from the corresponding sequence in bevacizumab by six amino acids; (3) each ranibizumab molecule has one binding site for VEGF (compared to bevacizumab's two); (4) ranibizumab is produced in prokaryotic *Escherichia coli*, and therefore it does not carry any glycosylation sites while bevacizumab is produced in a eukaryotic cell line (CHO cells) and is N-glycosylated in its Fc region; and (5) ranibizumab costs approximately 40 fold more than bevacizumab (\$2000 per injection compared to approximately \$50 per injection, respectively).

Ranibizumab was developed as an Fab fragment because it was thought that enhanced diffusion from the vitreous into the retina and choroid could be achieved with the smaller size molecule relative to full-length antibodies<sup>[19]</sup>. However, subsequent studies comparing the two suggest that the predicted size advantage may not translate into a therapeutic advantage in patients<sup>[20]</sup>. Affinity maturation of ranibizumab was also predicted to result in higher affinity binding to VEGF and increased biologic activity compared to bevacizumab<sup>[19]</sup>. Indeed, initial studies using the monovalent Fab-12 (from which the divalent bevacizumab was derived), suggested that the binding affinity of bevacizumab is markedly lower than what was later demonstrated for ranibizumab<sup>[21]</sup>. However, in a more recent study using a bivalent antibody (which reflects the two binding sites of bevacizumab), the dissociation constant ( $K_D$ ; inversely proportional to how tightly they bind to a molecule) of ranibizumab and bevacizumab for VEGF-A165 was roughly equivalent<sup>[22]</sup>.

A potential disadvantage of the Fc fragment is that bevacizumab may be more stable systemically than is ranibizumab; studies in animals appear to substantiate this prediction<sup>[23]</sup>. However, it remains unclear whether the low systemic levels of either bevacizumab or ranibizumab are sufficient to result in unwanted systemic effects. Additionally, reasonable disagreement remains as to whether a recent trial comparing bevacizumab to ranibizumab for the treatment wet AMD demonstrated a difference in the safety of the two therapies<sup>[20]</sup>. Nonetheless, some authors still argue that ranibizumab has been well studied in many randomized clinical trials with more long-term findings when compared with bevacizumab. Though this remains a hot topic of debate among retina specialists, the superiority of ranibizumab over bevacizumab has not been proven in clinical trials and both drugs are actively in use for treatment of VEGF driven retinopathies. A brief discussion comparing the two medications clinically is found below; extensive discussions on this topic can be found elsewhere<sup>[20]</sup>.

## RANIBIZUMAB IN DME

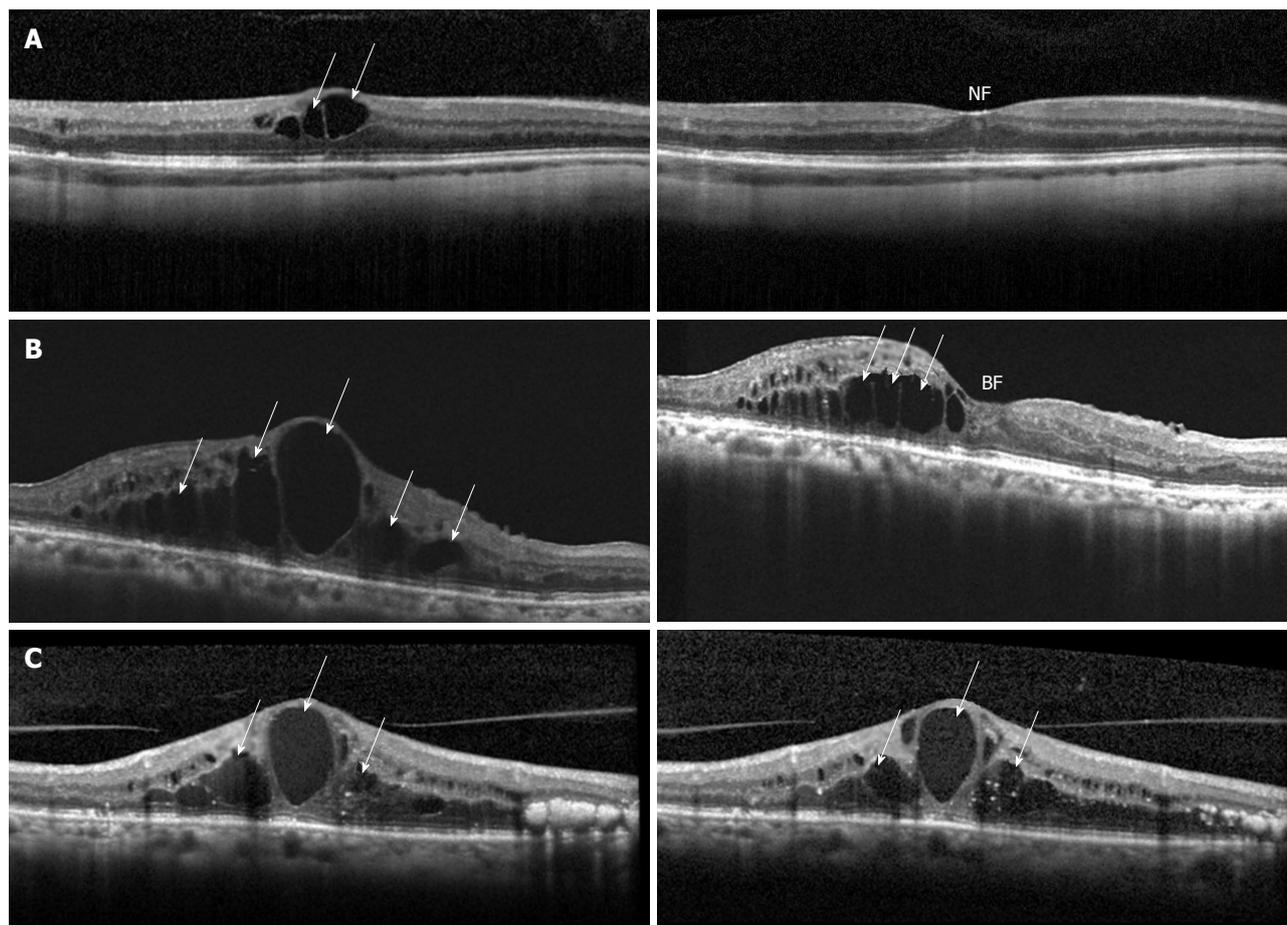
### DRCR trials

Several large clinical trials have investigated the role of

ranibizumab in the treatment of diabetic macular edema (Figure 3). The DRCR.net has conducted (and continues to conduct) large, multicenter, randomized clinical trials evaluating the treatment of diabetic eye disease. In an early study, the DRCR network demonstrated that approximately 30% of patients treated with laser photocoagulation gained two ETDRS lines of vision after 2 years following treatment, but up to 20% of these patients worsened by two ETDRS lines<sup>[24]</sup>. This led the DRCR to investigate additional treatment modalities. In a hallmark paper published in 2010, the DRCR showed that intravitreal injections of ranibizumab with prompt or deferred laser is more effective than prompt laser treatment alone for center involving DME<sup>[25]</sup>. In this study, 854 study eyes were randomized to sham injection with prompt laser, 0.5 mg ranibizumab with prompt laser, 0.5 mg ranibizumab with deferred ( $\geq 24$  wk) laser, or 4 mg triamcinolone with prompt laser. Sham or ranibizumab injections were given every four weeks up to week 12 and on a *pro re nata* (PRN or as needed) basis thereafter. In the two year follow up of this study, 29% of patients receiving ranibizumab plus prompt laser and 28% of patients receiving ranibizumab plus deferred laser had  $\geq 15$  letters of improvement, compared to 18% of patients in the prompt laser only group and 22% of patients in the triamcinolone plus prompt laser group gaining  $\geq 15$  letters. The mean change in visual acuity was a gain of 3 ETDRS letters for the prompt laser only group and a gain of 2 ETDRS letters in the triamcinolone plus prompt laser group. In contrast, the ranibizumab plus prompt laser group and the ranibizumab plus deferred laser group had a mean ETDRS letter gain of 7 and 9, respectively. At the three-year follow up<sup>[26]</sup>, the ETDRS letters gained in the ranibizumab plus prompt laser group was 6.8, and in the ranibizumab plus deferred laser group was 9.7. The percentage of patients with  $\geq 15$  letters of improvement was 26 and 32 in the ranibizumab plus prompt laser and ranibizumab plus deferred laser groups, respectively. Overall, this study clearly demonstrated that ranibizumab therapy (alone or in combination with laser) is superior to laser monotherapy. The suggestion that the ranibizumab plus prompt laser group may have slightly poorer visual outcomes than the ranibizumab plus deferred laser group will be investigated further by the DRCR.net in the longer term follow up of these patients.

### READ-2 trial

The first landmark study to demonstrate the efficacy of ranibizumab monotherapy was the READ-2 Study<sup>[27]</sup>. This prospective, multicenter trial randomized 126 patients to receive ranibizumab, combination ranibizumab and laser, or laser treatment alone in a 1:1:1 ratio. The ranibizumab group received 0.5 mg ranibizumab at baseline and months 1, 3 and 5. The laser group received laser photocoagulation at baseline and month three if needed, and the combination group received 0.5 mg ranibizumab and laser at baseline and month three. The primary endpoint was 6 mo, but patients were followed to 24 mo.



**Figure 3** High resolution optical coherence tomography demonstrating different responses to treatment of diabetic macular edema patients with ranibizumab. A: Modest cystoid macular edema (CME) with few inner retinal cysts (white arrows) and loss of the foveal contour (left) which completely resolved with return of a normal foveal contour (NF) and excellent vision one month after a single injection of ranibizumab (right); B: Massive CME with numerous inner and outer retinal cysts (white arrows) with complete loss of the foveal contour (left) which partially resolves resulting in a blunted but improved foveal contour (BF) and a significant improvement in vision following treatment with ranibizumab (right); C: Massive CME with numerous inner and outer retinal cysts (white arrows) with complete loss of the foveal contour (left) which does not respond despite repeated treatment with ranibizumab (right). The last response is uncommon; this patient was ultimately treated with intraocular steroids, and did have a sustained improvement of the edema and a modest improvement in vision.

After the primary endpoint of 6 mo, all patients were eligible to receive additional ranibizumab, and patients in the laser or combination group were also eligible to receive additional laser treatments. At the primary endpoint of 6 mo, the mean number of ETDRS letters gained in the ranibizumab group was 7.24 letters, in the combination group was 3.80 letters, and in the laser alone group there was a mean reduction of 0.43 ETDRS letters. In the long term follow up at 24 mo, the laser group had an increase of 5.1 letters, the increase in the ranibizumab group (7.7 letters) and the combination group (6.8 letters) was higher, though not significantly so. At the 24-mo point, 24% of patients in the ranibizumab monotherapy group gained  $\geq 15$  ETDRS letters compared with 18% of the laser monotherapy patients and 26% of the combination therapy patients. This trial nicely complemented the DRCR trial, as it demonstrated that long-term improvements in visual acuity could be achieved with ranibizumab monotherapy.

### RESOLVE and RESTORE

The DRCR and READ studies examined the therapeutic

potential of ranibizumab for the treatment of DME in the United States. It was anticipated that the efficacy of ranibizumab could be extrapolated to populations outside of the United States; this assumption has been supported by several international clinical trials. The RESOLVE study was a smaller, multi-center, sham controlled trial which randomized 151 patients to receive either sham, ranibizumab 0.3 mg, or ranibizumab 0.5 mg injections, monthly for three months followed by PRN treatment<sup>[28]</sup>. Rescue treatment with laser was permitted if necessary. This study also allowed for “dose doubling” at the discretion of the investigator; thus, after month one, patients in the 0.3 mg ranibizumab group were eligible to receive 0.6 mg, and patients in the 0.5 mg group were eligible to receive 1.0 mg. At the 12 mo follow up, the pooled ranibizumab group had an average gain of 10.3 ETDRS letters compared to an average decline of 1.4 letters in the sham group. The percentage of patients gaining  $\geq 15$  ETDRS letters was 33% and 5% for the pooled ranibizumab and sham groups, respectively. These numbers are similar to that found in the READ-2 study. It is unclear how to interpret the variable dosing, and no clear guidelines can be

deduced from the dosing scheme.

The RESTORE study group examined patients from 10 European countries<sup>[29]</sup>. This study randomized 345 patients to one of three treatment groups: ranibizumab 0.5 mg injection monotherapy, laser monotherapy, or ranibizumab plus laser combination therapy. Patients in either the ranibizumab monotherapy or combination group received three initial consecutive monthly injections, followed by PRN monthly injections through month 11. Laser monotherapy or combination patients received initial laser treatment either in one or two sessions, followed by re-treatment every three months if necessary. The investigators found that at the primary endpoint (12 mo), the average gain in ETDRS letters in the ranibizumab monotherapy group was 6.1, in the combination group was 5.9, and in the laser monotherapy group was 0.8. At the 12-mo endpoint, the percentage of patients who gained  $\geq 15$  letters was 26, 27, and 9 for the ranibizumab monotherapy, combination therapy, or laser monotherapy groups, respectively. These percentages are consistent with those found in the other studies discussed above. The percent of patients gaining  $\geq 15$  letters with laser monotherapy is lower in this study compared with the DRCR and READ-2 studies, which may reflect the fact that this study only followed patients out to one year. Overall, this study supports the findings of the studies discussed above.

### **RISE and RIDE**

More recently, results from the RISE and RIDE studies were published<sup>[30]</sup>. These two parallel, phase 3, multicenter, sham controlled studies randomized patients to sham injections, or injections with 0.3 or 0.5 mg ranibizumab, on a monthly basis for 24 mo. Patients were eligible for laser rescue treatment if treatment criteria - as established by the study investigators - were met. The RISE study enrolled 377 patients, whereas the RIDE study enrolled 382 patients. The patients in each study were randomized 1:1:1 to each of the three treatment groups. In the RISE study, at the 24-mo end point, 39.2% of patients receiving monthly ranibizumab at a 0.5 mg dose, and 44.8% of patients receiving monthly ranibizumab at a 0.3 mg dose gained  $\geq 15$  ETDRS letters, compared to 18.1% of patients receiving sham injections. Similarly, in the RIDE trial, the percentage of patients gaining  $\geq 15$  ETDRS letters in the 0.5 and 0.3 mg ranibizumab dose was 45.7 and 33.6, respectively, compared with 12.3% in the sham laser group. The studies were not powered to compare the two doses of ranibizumab, but were powered to show significance compared with sham injections.

From the RISE and RIDE studies, it appears that further improvements in visual acuity can be achieved with monthly dosing of ranibizumab rather than PRN dosing. However, it is not clear whether monthly injections will result in a further improvement in vision in an individual who responds well to PRN dosing, or whether a subgroup of patients requires monthly injections to achieve a significant improvement in vision. Ultimately, the ad-

ditional improvement in visual acuity in an individual patient must be balanced with the theoretical and known safety concerns associated with an increase in the frequency of intravitreal injections of ranibizumab. Though the treatment and dosing varied among the different trials, collectively, these trials demonstrate that ranibizumab therapy (alone or in combination with laser therapy) results in improved visual acuity outcomes than does laser monotherapy.

### **SAFETY**

Each of these trials also reported safety data. In each study, the incidence of ocular adverse events, as well as serious adverse events such as stroke or heart attack were rare. The biggest ocular concern is endophthalmitis. In the RISE and RIDE studies there were four total cases of endophthalmitis out of 500 patients in the two-year follow up of the study (0.8%; 1 in RISE with 0.3 mg ranibizumab, 3 in RIDE, 1 from 0.3 mg group and 2 from 0.5 mg group). The three year follow up of the DRCR study reported a total of 3 cases of endophthalmitis out of 375 (also 0.8%) patients receiving ranibizumab injections, in either the prompt or deferred laser groups. The RESTORE study had no cases of endophthalmitis. RESOLVE had 2 cases of endophthalmitis out of 102 injection patients (2%) over the year of the study. The average number of injections per patient varied in all of these studies, but generally speaking, the rate of endophthalmitis was similar among the trials, and risk of endophthalmitis with an individual injection was extremely low based on these numbers.

The major systemic safety concern with anti-VEGF treatment is thromboembolic events. In the one-year RESTORE study there were 6 arterial thromboembolic events (5.2%) in the ranibizumab (0.5 mg) group, whereas only one such event occurred in the laser group and the laser plus ranibizumab group. The group sizes were similar, and the analysis did not support a statistical difference between ranibizumab treated groups and the laser only group. The one-year RESOLVE study also reported a low incidence of arterial thromboembolic events with no significant difference among treatment groups (3 of 102 in ranibizumab groups, 2 of 49 in sham group). The two-year follow up of the DRCR study also reported no significant difference in thromboembolic events in ranibizumab or sham treated groups. There was a trend, however, for a decreased number of such events in the ranibizumab treated groups. Non-fatal CVAs occurred in 8 out of 130 (6%) sham injection patients, and in 7 out of 375 (2%) ranibizumab treated patients. The total number of cardiovascular events was 17 out of 130 (13%) in the sham injection patients, and 25/375 (7%) in the ranibizumab treated patients.

In the RISE and RIDE studies, thromboembolic events and deaths were similar between sham and treatment groups. These studies did report that the number of deaths and CVAs were numerically higher in the ra-

nibizumab groups compared to sham groups, with the highest incidences of CVA and death being in the ranibizumab 0.5 mg group. The number of CVAs in the RISE and RIDE studies combined were 4 out of 250 (1.6%), 3 out of 250 (1.2%), and 8 out of 250 (3.2%), in the sham, 0.3 mg, and 0.5 mg groups, respectively. The number of deaths in the combined studies was 3 out of 250 (1.2%), 7 out of 250 (2.8%), and 11 out of 250 (4.4%) in the sham, 0.3 mg, and 0.5 mg groups, respectively. At this time, the trend towards increased CVA and death in the higher ranibizumab group should be acknowledged but interpreted with caution. Longer follow up studies will be necessary to substantiate any increased risk of stroke with ranibizumab treatment.

## COMBINATION TREATMENTS AND INJECTION FREQUENCY

The long term follow up from the DRCR.net clinical trial raises the possibility that combination therapy with ranibizumab and laser treatments may not be as effective as ranibizumab monotherapy alone for the treatment of DME. In clinical practice, many physicians continue to employ focal laser as part of the treatment for these patients. One theoretical advantage of combination treatment is that it may decrease the frequency and total number of required intravitreal ranibizumab injections. In the DRCR studies discussed above, the median number of ranibizumab injections during the first year was 8 and 9, in the ranibizumab plus prompt or deferred laser, respectively, out of a maximum of 13. Between years 1 and 2, the median number of injections was 2 and 3, in the prompt and deferred laser groups, out of a maximum of 13. In year three, the median number of injections decreased to 1 and 2, in the prompt and deferred laser groups, respectively. This study suggests that initiating prompt laser or deferring laser does not significantly affect the number of injections needed, although the trend is towards less total injections in the prompt laser group. However, this decrease in number of injections in the prompt laser groups was speculated to contribute to the slightly worse visual acuity outcomes in three year follow up<sup>[26]</sup>.

The data regarding number of injections in the READ-2 study is difficult to interpret because the frequency of evaluation varied between the two groups (every two months for the injection only group and every three months for the combination group). Perhaps the most straightforward comparison can be made from the RESTORE study in which both ranibizumab monotherapy patients and combination therapy patients were evaluated monthly and eligible for additional ranibizumab injections monthly. At the 12-mo follow up, the ranibizumab monotherapy group and the combination group received a mean of 7 and 6.8 injections, with a median of 7 and 7 injections, respectively. This data from the RESTORE study suggests that combination therapy with laser and ranibizumab does not reduce the number of

required injections. However, it is not known what the results would be if these patients were followed to two years and beyond. Based on the current follow up data from clinical trials, the role of focal laser in the treatment of diabetic macular edema in the anti-VEGF era is not well delineated.

## OTHER ISSUES FOR CONSIDERATION

The wealth of data from the clinical trials described here strongly suggests that ranibizumab, (and likely other effective anti-VEGF therapies) are beneficial for the treatment of DME. The safety data from these studies suggest that ranibizumab injections, including monthly injections for two years, are well tolerated and safe. It also appears that treatment of DME with ranibizumab may have additional benefits in patients with diabetic eye disease. The progression of diabetic retinopathy in patients treated with monthly injections with ranibizumab is significantly lower than those treated with laser photocoagulation<sup>[31]</sup>. This was not surprising since VEGF is also known to play a fundamental role in the development and progression of diabetic retinopathy. The use of ranibizumab to treat diabetic retinopathy is discussed elsewhere<sup>[31]</sup>. The contribution of anti-VEGF therapy to the promotion or inhibition of peripheral retinal and macular ischemia remains unclear; a comprehensive recent review of this topic can be found elsewhere<sup>[32]</sup>.

What is also not clear is whether patients who fail treatment with one anti-VEGF approach would be sensitive to another approach. Certainly there are patients who develop tachyphylaxis after the use of either ranibizumab or bevacizumab; and these patients may respond well to the other anti-VEGF drug. However, given the similarities between these two medications, it would seem unlikely that an individual patient would be sensitive to one treatment but not the other. No large studies directly comparing bevacizumab to ranibizumab have been published in the setting of diabetic macular edema, but a large study directly comparing the two medications for the use of neovascular age-related macular degeneration suggested that the medications are probably equivalent for this disease<sup>[20]</sup>. Nonetheless, this remains a contentious issue.

The use of bevacizumab for diabetic macular edema was investigated in the BOLT trial<sup>[18]</sup>. The purpose of this study was to compare bevacizumab therapy to macular laser therapy for diabetic macular edema. The bevacizumab arm patients received three injections, six weeks apart, followed by PRN treatment every six weeks. The results showed that after two years, bevacizumab treatment was superior to focal laser treatment. Patients in the bevacizumab arm gained a mean of 8.6 ETDRS letters, and those in the laser arm lost a mean of 0.5 letters. In the bevacizumab arm, 32% of patients gained at least 15 ETDRS letters, and the median number of injections was 9 during the first year, and 4 during the second year. Although the results cannot be directly compared to the

ranibizumab studies due to differences in the specifics of the study design and criteria, the changes seen with bevacizumab are of similar magnitude as the changes in the ranibizumab studies. Given the similarities between bevacizumab and ranibizumab, the introduction of other anti-VEGF therapies (*e.g.*, aflibercept or Eylea®) may be a better alternative for patients who fail treatment with ranibizumab or bevacizumab<sup>[33]</sup>; the use of intravitreal aflibercept for the treatment of DME is discussed elsewhere in this review series.

A final issue that remains under debate is the cost-effectiveness of ranibizumab compared to bevacizumab. Despite the similarities between these two medications, there is a significant difference in the cost between ranibizumab and bevacizumab. It is not clear whether the cost difference reflects a difference in safety or efficacy of these two medications. Several analyses have been performed exploring this question; a comprehensive recent review of this topic can be found elsewhere<sup>[34]</sup>.

## CONCLUSION

There remains little doubt that ranibizumab has an important role in the treatment of DME. Current studies are now focused on fine-tuning the recommended treatment regimen to determine the most effective treatment dose, frequency, and duration to optimize visual outcomes and safety. Additional efforts to extend the interval between treatments or to identify populations of patients who require less frequent follow up or injections are also underway. The results from these studies may reduce the risks of monthly injections, and the burden on many patients tethered by their required monthly visits to their ophthalmologists.

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## Enzymatic vitrectomy for diabetic retinopathy and diabetic macular edema

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### Abstract

The aim of this paper is to determine the role of enzymatic vitrectomy performed by intravitreal injection of autologous plasmin enzyme (APE) in the management of diabetic retinopathy and diabetic macular edema (DME). Diabetic patients with proliferative diabetic retinopathy or DME and evident posterior hyaloid adherence to the retinal surface were included. All cases were treated with an initial intravitreal injection of APE and reevaluated one month later, measuring changes in best-corrected visual acuity (BCVA), macular thickness and the status of the posterior hyaloid. A second APE injection was performed in cases with no evident posterior vitreous detachment (PVD) after the initial treatment. Sixty-three eyes were included in the present review. A complete PVD appeared in 38% of cases (24 eyes) after one injection of plasmin and the total increased to 51% (32 eyes) after the second injection, separated at least by one month. The central macular

thickness improved in all cases (100%) and BCVA in 89%. Finally, in 50% of eyes with proliferative diabetic retinopathy, a high reduction of new vessels regression was observed. Enzymatic vitrectomy could be considered a good therapeutic alternative in diabetic retinopathy and macular edema.

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**Key words:** Enzymatic vitrectomy; Autologous plasmin; Diabetic macular edema; Diabetic retinopathy

**Core tip:** Diabetes mellitus might be considered as a pandemic disease with its incidence and prevalence increasing exponentially, even becoming epidemic. The aim of the present review was to analyze the clinical efficacy of the intravitreal injection of autologous plasmin enzyme in the treatment of diabetic retinopathy and diabetic macular edema and to determine the role of enzymatic vitrectomy as a therapeutic approach in such cases.

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### INTRODUCTION

Diabetes mellitus (DM) causes diabetic retinopathy (DR) and diabetic macular edema (DME) as a consequence of diabetic microangiopathy. Nowadays, DM might be considered as a pandemic disease with its incidence and prevalence increasing exponentially, even becoming epidemic. The prevalence of DR increases with the time of

evolution of the systemic disease, with nearly 100% of patients showing some degree of diabetic retinopathy after 20 years of evolution. On the other hand, DME develops in 14%-25% of patients after 10 years of DM<sup>[1-3]</sup>. DR is a major cause of visual loss and a leading cause of blindness, whereas DME is the most common cause of visual loss in people under 50 years of age in developed countries<sup>[4,5]</sup>.

Retinal neovascularization is a significant risk factor for severe visual loss in patients with DM, with optic disk new vessels as the maximum expression of such risk<sup>[6]</sup>. Laser photocoagulation has been the mainstay of treatment for DME and DR, but only 60% of patients with proliferative diabetic retinopathy (PDR) respond to panretinal photocoagulation, with regression of the neovascularization within 3 mo<sup>[2]</sup>. Laser photocoagulation mainly preserves vision rather than restoring it in cases of DME and PDR<sup>[7-9]</sup>.

More recently, new therapeutic approaches have been developed for the management of both PDR and DME, including intravitreal injections of steroids (triamcinolone, sustained release intravitreal corticosteroid implant) or vascular endothelial factor inhibitors (VEGF), such as pegaptanib (Macugen; OSI pharmaceuticals, Melville, NY), bevacizumab (Avastin; Genentech, San Francisco, CA), ranibizumab (Lucentis; Genentech, San Francisco, CA) and aflibercept (Eylea, Regeneron Pharmaceutical, Inc, Tarrytown, NY), achieving vision improvement in a significant number of patients<sup>[10-12]</sup>.

The incidence of DME and the progression rate of PDR are significantly lower in patients with spontaneous or surgical posterior vitreous detachment (PVD). It has been demonstrated that the adherence of the posterior hyaloid to the inner limiting membrane plays an important role in the development of DME and also in the growth of new vessels and its consequences, vitreous hemorrhage and tractional retinal detachment<sup>[13-17]</sup>.

Enzymatic vitrectomy or pharmacological vitreolysis by intravitreal injection of autologous plasmin enzyme (APE) has been proposed as an effective neoadjuvant treatment for vitreous surgery by facilitating the surgical detachment of the posterior hyaloid and vitreoretinal membranes<sup>[18-20]</sup>.

Plasmin, a serine protease, is active against laminin and fibronectin, located in the interface between the posterior vitreous cortex and the internal limiting membrane, and is responsible for the attachment of the vitreous to the retinal surface<sup>[21]</sup>.

The aim of the present review was to analyze the clinical efficacy of the intravitreal injection of APE in the treatment of DR and DME and to determine the role of enzymatic vitrectomy as a therapeutic approach in such cases.

## RESEARCH

The primary outcome was considered as the proportion

of patients with a complete PVD following intravitreal injection of APE. The secondary outcomes were: number of patients with improvement in visual acuity and/or central macular thickness measured by optical coherence tomography (OCT); regression of new vessels in cases of PDR; and safety assessment of intravitreal injection of APE.

Before the injection of APE, all patients underwent a comprehensive ocular examination, including best corrected visual acuity (BCVA), slit-lamp examination, tonometry, indirect ophthalmoscopy, macular thickness measurement and assessment of posterior hyaloid status by OCT, color photographs and fluorescein angiography.

### APE injection

A total volume of 0.2 mL of APE was obtained by a simplified method<sup>[21]</sup> and prepared 45 min prior to injection, sterilized through a 0.22  $\mu$ m pore filter. Patients received unilateral intravitreal injections of APE through pars plana using a 30-gauge needle according to the routine procedure of intravitreal injections.

## TREATMENT

A total of 63 eyes of 52 patients were included in the present study. Of them, 10 eyes showed PDR with optic disk new vessels and 53 eyes showed DME (33 refractory DME cases and 20 treatment-naïve patients).

### Primary outcome: posterior vitreous detachment

A complete PVD appeared in 38% of cases (24 eyes) after one injection of plasmin and the total increased to 51% (32 eyes) after the second injection, separated at least by one month.

### Secondary outcomes

Central macular thickness decreased in all cases with DME, even in cases without PVD following intravitreal injections of APE. BCVA improved in 89% of the treated eyes (56) (Figures 1, 2).

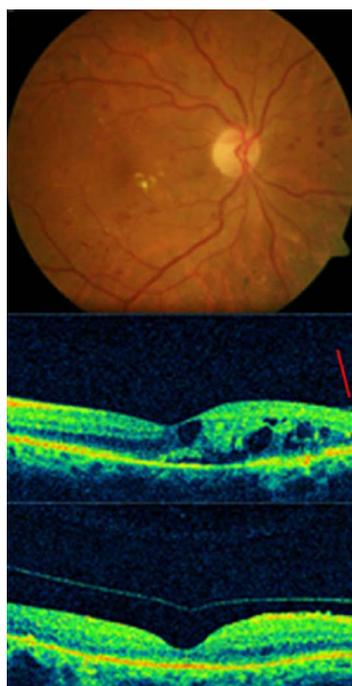
In cases with PDR, a highly significant regression in the new vessel area was observed in 5 cases (50%) (Figure 3); in 2 cases a partial regression was evidenced (20%), whereas the 3 remaining cases did not experience any change (30%).

### Safety profile

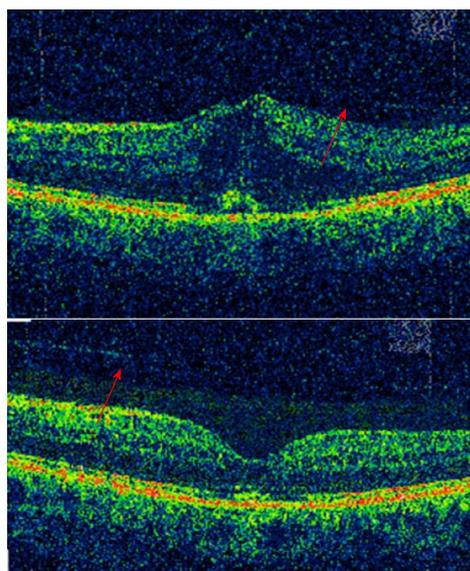
The treatment was well tolerated in all cases. No side effects were evidenced through the follow-up (uveitis, vitreous hemorrhage, cataract, ocular hypertension or retinal detachment).

## DISCUSSION

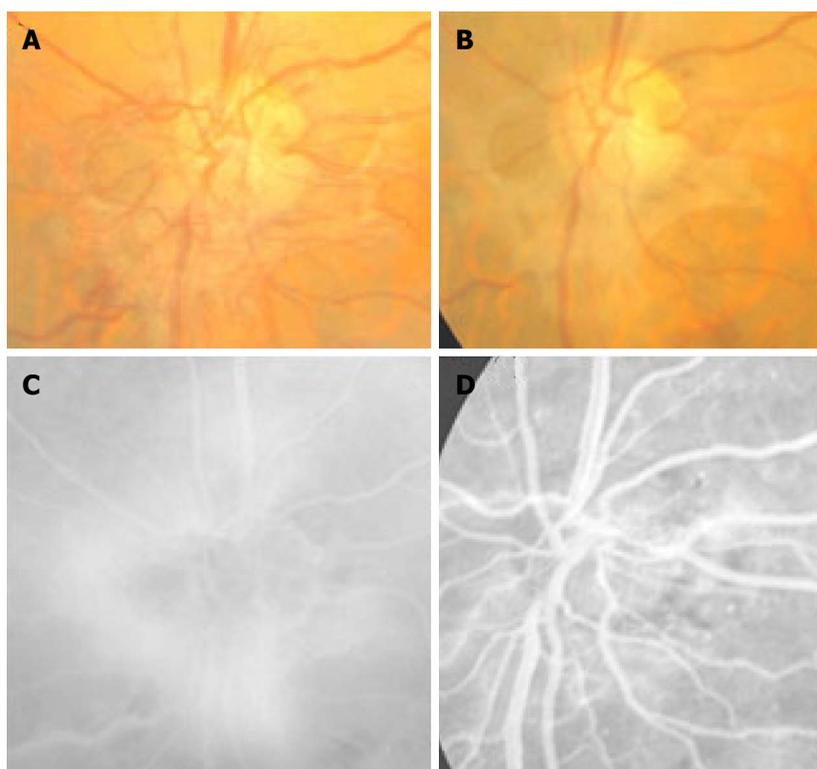
The analyzed data suggested a possible role for enzymatic vitreolysis by intravitreal injection of APE for the management of PDR and DME. The efficacy of this



**Figure 1** Complete posterior vitreous detachment and complete resolution of macular edema in a diabetic patient treated with autologous plasmin.



**Figure 2** Optical coherence tomography showing a diffuse and refractory diabetic macular edema treated with autologous plasmin. Red arrows mark the posterior hyaloid before and after treatment.



**Figure 3** Regression of the new vessels of the optic disc in a treated eye with plasmin. A, B: Color images before and after treatment; C: Fluorescein angiogram shows the active vessels; D: The important but not complete resolution of leakage.

procedure is related to the biochemical modification of the vitreous composition induced by the enzymatic separation or weakening of the union between the posterior vitreous cortex and the internal limiting membrane, leading to the development of PVD, and the liquefaction of the vitreous gel<sup>[22]</sup>. This procedure is dynamic with an important impact on the vitreous cavity. PVD not only has a protective role in a variety of retinal disorders, but also induces changes in the intraocular concentration of

molecules, including VEGF, and increases intravitreal molecular diffusion coefficients and intravitreal oxygen levels<sup>[23-26]</sup>; this could also contribute to the good results obtained with this therapy.

APE employed in the present study was obtained by a simplified method and can be prepared in the operating room 45 min prior to the intravitreal injection. This constitutes a significant advantage compared to previously described techniques that required a longer and more

expensive procedure. On the other hand, the method we propose has a wide variability and we cannot exactly recognize the concentration of APE that is injected and it depends on the plasminogen levels in each patient<sup>[27-30]</sup>.

We achieved an overall efficacy of 50% in terms of inducing PVD in cases of DME and PDR, without any significant ocular side effects.

In conclusion, enzymatic vitrectomy performed by the intravitreal injection of APE might be effective and could be considered as an alternative for diabetic patients before performing other treatments, such as intravitreal injections of anti-VEGF or steroids, surgical vitrectomy or laser. Further studies are necessary to assess the efficacy and safety for the longer term and most convenient therapeutic strategy, including association of treatments, in diabetic patients to get better and more permanent results.

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## Diabetic macular edema: New promising therapies

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### Abstract

The treatment of diabetic macular edema is rapidly evolving. The era of laser therapy is being quickly replaced by an era of pharmacotherapy. Several pharmacotherapies have been recently developed for the treatment of retinal vascular diseases such as diabetic macular edema. Several intravitreal injections or sustained delivery devices have undergone phase 3 testing while others are currently being evaluated. The results of clinical trials have shown the superiority of some of these agents to laser therapy. However, with the availability of several of these newer agents, it may be difficult to individualize treatment options especially those patients respond differently to various therapies. As such, more effort is still needed in order to determine the best treatment regimen for a given patient. In this article, we briefly summarize the major new therapeutic additions for the treatment of diabetic macular edema and allude to some future promising therapies.

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**Key words:** Diabetic macular edema; Diabetic retinopathy;

Intravitreal injection; Laser photocoagulation; Pharmacotherapy; Sustained release drug delivery devices; Vitrectomy

**Core tip:** This manuscript concisely reviews most of the new and emerging therapies for diabetic macular edema. It focuses on therapies that have been tested through clinical trials, but also includes therapies that have not. The article focusses on laser therapy, intravitreal anti-vascular endothelial growth factor agents, intravitreal triamcinolone and newer steroid implants, and vitrectomy. It also briefly discusses several other therapeutic approaches that may be available in the near future.

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### INTRODUCTION

Diabetic macular edema (DME) is a common manifestation of diabetic retinopathy that causes loss of central vision<sup>[1]</sup>. Data from the Wisconsin Epidemiologic Study of Diabetic Retinopathy stated that the 10-year cumulative incidence of macular edema was 20.1% among those with type 1 diabetes and 25.4% among people with type 2 diabetes treated with insulin<sup>[2]</sup>. The ultimate treatment for DME involves strict glycemic and blood pressure control, which was demonstrated by the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study<sup>[3-5]</sup>. Additionally, laser treatment for clinically significant macular edema (CSME) has been the mainstay form of treatment after the recommendations by the Early Treatment Diabetic Retinopathy Study (ETDRS) study that demonstrated a 50% reduction in moderate visual loss following focal laser photocoagulation<sup>[6,7]</sup> with only 3% of patients improving > 3 lines of

visual acuity (VA) 36 mo after treatment. Therefore, a variety of other therapies have been studied with the aim of improving vision in more patients as well as preventing deterioration of VA in most. These include surgical options, intravitreal corticosteroids, novel intravitreal vascular endothelial growth factor (VEGF) inhibitors and other various new pharmacotherapies that are currently being investigated. Results from new clinical trials are constantly being published, challenging frequent updates on the most recent therapeutic options for this complex disease. In this report, we discuss the evolution of DME treatment over the past few years and give general guidelines for treatment based on available evidence to date.

## LASER PHOTOCOAGULATION

Until the advent of intravitreal anti-VEGF agents, laser photocoagulation has been unequivocally the standard of care for treatment of DME<sup>[1]</sup>. The efficacy of focal laser treatment may in part be due to occlusion of leaking microaneurysms, but the exact mechanisms by which focal photocoagulation reduces DME is unknown. Histopathological studies show changes located in the retina and retinal pigment epithelium (RPE)<sup>[8,9]</sup>. Some researchers have suggested that with reduced retinal tissue following photocoagulation, autoregulation results in decreased retinal blood flow to the macula with lower fluid flow resulting in decreased edema<sup>[10]</sup>. Others have hypothesized that the reduced retinal blood flow is due to improved oxygenation following photocoagulation. Biochemical and physiological studies propose that resolution of the edema may also result from changes in the biochemical processes within the RPE<sup>[11-17]</sup>. An indirect effect of retinal photocoagulation on macular edema has also been supported by the effectiveness of grid treatment alone without direct focal treatment of microaneurysms<sup>[18,19]</sup>.

The ETDRS trial (1985)<sup>[6]</sup>, was the first properly performed multicenter, randomized trial to determine the benefits of laser for DME. Three years after randomization, patients treated with focal photocoagulation for CSME had a 50% reduction in the risk of moderate visual loss compared to controls (from 24% to 12%). However a gain of 3 or more lines during the same period was only reported in three percent of the patients.

The suggested guidelines for laser photocoagulation for DME as provided by the ETDRS<sup>[6]</sup> is a direct treatment to leaking microaneurysms and grid treatment to diffuse macular leakage and areas of non-perfusion in thickened retina in cases of non-proliferative diabetic retinopathy (NPDR). Because initial pan-retinal photocoagulation (PRP) may worsen macular edema by increasing the inflammatory component and/or central retinal blood flow<sup>[20,21]</sup>, the ETDRS recommended combination of PRP and focal laser photocoagulation for DME in general for selected cases of severe NPDR and early proliferative diabetic retinopathy (PDR). The ETDRS also suggested that if the retinopathy severity allows, eyes with CSME that need PRP may benefit from treatment

of the macular edema first followed by PRP 2-4 wk later. Eyes with high risk PDR and CSME on the other hand may be better managed by concomitant treatment of the macular edema in addition to PRP in the same setting because such eyes are at higher risk of bleeding and severe visual loss.

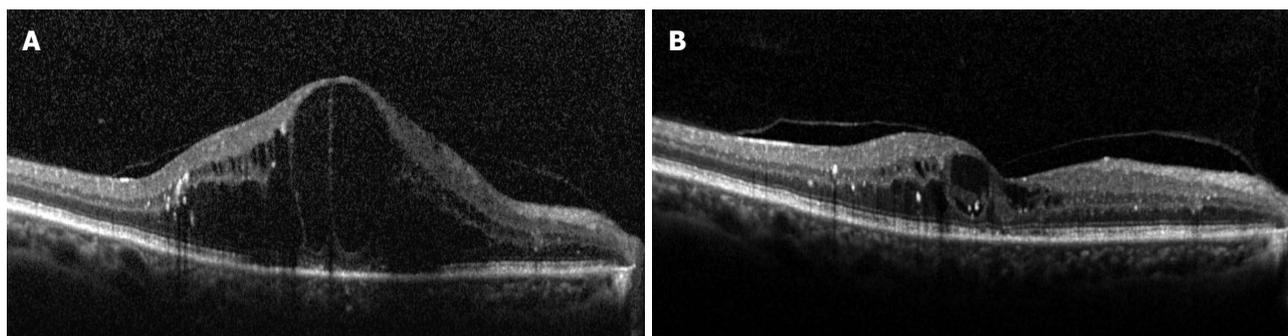
Although effective, the conventional ETDRS macular photocoagulation protocol causes visible laser scars that can enlarge following treatment and encroach towards fixation<sup>[22-24]</sup>. In addition, the thermal effects of laser photocoagulation for DME can lead to complications, such as choroidal neovascularisation (CNV)<sup>[25,26]</sup>, subretinal fibrosis<sup>[27,28]</sup> and visual field loss (*i.e.*, central and paracentral scotomata)<sup>[29]</sup>. Consequently, this intrinsic damage from visible end point laser photocoagulation has prompted interest of many retinal specialists to treat by reducing laser exposure duration and using a sub-visible clinical end point than originally specified in the ETDRS<sup>[30-32]</sup>. However, exact evidence to support such practice is still deficient. Recently, the outcomes following modified ETDRS laser protocol and mild macular grid (MMG) laser photocoagulation strategies were evaluated in a randomized controlled trial including 263 eyes with previously untreated DME that were followed for over 12 mo period. The MMG consisted of sub-threshold (non-visible) laser applications in the form of a grid treatment. Though, there was a significantly greater reduction in macular thickness in the modified ETDRS laser protocol group, there was no difference in the mean change in best-corrected VA (0 letters in ETDRS and -2 letters in the MMG group,  $P = 0.10$ ). The conclusion of this trial suggested that modified ETDRS focal photocoagulation should continue to be the standard approach for treating DME<sup>[33]</sup>.

## INTRAVITREAL TRIAMCINOLONE ACETONIDE

In recent years, several studies<sup>[34-37]</sup> have demonstrated the possible benefits of intravitreal injection of triamcinolone acetonide (IVTA) in the management of refractory DME; especially that IVTA does not appear to have a retinotoxic effect<sup>[38]</sup>. Triamcinolone acetonide (TA) is a synthetic steroid of the glucocorticoid family with a molecular weight of 434.50. Its decreased water solubility accounts for its prolonged duration of action<sup>[39]</sup>. The elimination half-life of the commercial preparation of TA in the vitreous humor of rats was recently reported by Oishi *et al*<sup>[40]</sup> as 6.08 d. In nonvitrectomized patient eyes, the mean elimination half-life was 18.6 d, while in 1 post vitrectomy patient it decreased to 3.2 d<sup>[41]</sup>.

The precise mechanism of action of corticosteroids is still unknown, however, the rationale could be found in their ability to inhibit arachidonic acid pathway, of which prostaglandin is a product.

In addition to the anti-inflammatory effect, TA increases the levels of tight-junctions in between endothelial cells<sup>[42]</sup> and thus lessens vessel leakage. It also has an angiostatic action through VEGF inhibition<sup>[43]</sup> and



**Figure 1** Horizontal spectral-domain optical coherence tomography scans of the macula before (A) and after (B) 1 mg of intravitreal triamcinolone therapy in a patient with diabetic macular edema in the right eye. Four months following treatment, the macular edema almost completely resolved (B). The Snellen visual acuity improved from 20/200 to 20/60.

therefore may have a useful effect on DME. IVTA has been increasingly used in refractory DME and even as an alternative to macular photocoagulation, although its use is technically off-label for DME.

In recent years many authors have investigated the possible benefits of intravitreal TA for treating DME. The most considerable evidence reported by Sutter *et al.*<sup>[36]</sup>, in a prospective, double-masked, and randomized trial comparing 4-mg intravitreal TA with placebo. This study reported that 55% of 33 eyes treated with 4 mg of IVTA improved by 5 or more letters of best-corrected visual acuity (BCVA) at 3 mo compared with 16% of 32 eyes treated with placebo ( $P = 0.002$ ). Macular edema was decreased by 1 or more grades as determined by masked semi-quantitative contact lens examination in 25 of 33 treated eyes (75%) *vs* 5 of 32 untreated eyes (16%;  $P < 0.0001$ ).

Several studies were performed to compare the efficacy and safety between IVTA and other modalities of treatment (*e.g.*, focal/grid photocoagulation<sup>[44-47]</sup>, vitrectomy with internal limiting membrane peeling<sup>[48]</sup> and intravitreal bevacizumab<sup>[49]</sup>). The Diabetic Retinopathy Clinical Research network (DRCRnet)<sup>[44]</sup> group designed a two year multicenter randomized clinical trial to compare IVTA (1- and 4-mg doses) with modified ETDRS focal/grid photocoagulation for the treatment of DME. The mean VA was better in the 4-mg IVTA group at 4 mo than in either the laser or the 1-mg IVTA groups; though this beneficial effect was not maintained. At 2 years after starting the treatment, the mean VA was statistically better in the laser group compared to the steroid-injected groups. Optical coherence tomography (OCT) results generally paralleled the VA results. The 3-year visual outcome results of the DRCRnet were consistent with the previously published 2-year results. Furthermore, the cumulative probability of cataract surgery by 3 years was most frequent in the 4-mg IVTA group (83%) *vs* the 1-mg IVTA group (46%) and the laser group (31%). The intraocular pressure (IOP) increased by more than 10 mmHg at any visit in 4%, 18% and 33% of the eyes, in the laser, 1 mg IVTA and 4 mg IVTA groups respectively. This randomized study pointed out clearly that focal/grid photocoagulation is a better treatment option than IVTA

in eyes with DME involving the center of the macula with VA between 20/40 and 20/320. The DRCR net<sup>[50]</sup> reported another randomized clinical trial evaluating the efficacy of intravitreal ranibizumab (RBZ) with prompt or deferred laser treatment, the combination of IVTA with prompt laser photocoagulation, and laser treatment alone. The 1-year mean change ( $\pm$  standard deviation) in the VA letter score from baseline was significantly greater in both RBZ groups but not in the IVTA + prompt laser group ( $+4 \pm 13$ ,  $P = 0.31$ ) compared with the laser group ( $+3 \pm 13$ ). Nevertheless, among a subgroup of 62 pseudophakic eyes, VA results were considerably better than for phakic eyes at baseline in the IVTA + prompt laser group such that the degree of improvement appeared comparable to that of the pseudophakic eyes in the RBZ groups and superior to that of the pseudophakic eyes in the sham + prompt laser group at 1 year and 2 years.

The appropriate dose of IVTA for DME is a controversial topic. As reported by Hauser *et al.*<sup>[51]</sup> and Audren *et al.*<sup>[52]</sup> the use of the higher 4-mg dose of IVTA does not have enough advantages over the lower 1- or 2-mg doses. However, Lam *et al.*<sup>[53]</sup> showed that the higher dose (8 mg *vs* 4 mg) had a more persistent effect on both VA and central macular thickness (CMT) though associated with more ocular hypertensive responses.

Despite having the potential for benefit in selected patients (Figure 1), IVTA does carry substantial risks which are related to both the injection itself and the potential toxicity of corticosteroids. Injection-related events include endophthalmitis, vitreous hemorrhage, and retinal detachment. The potential toxicity of corticosteroids includes the development of cataracts<sup>[54]</sup> and glaucoma<sup>[55]</sup>. Moreover, the treatment effect typically wanes, and patients may require repeated injections that increases the chances for the above-mentioned risks, and therefore, the benefits of its use have to be weighed against the risks.

## VEGF ANTAGONISTS

VEGF levels are elevated in the retina and the vitreous of eyes with diabetic retinopathy<sup>[56]</sup>. Also, when VEGF was injected in mouse eyes it was found to cause breakdown of the inner blood retinal barrier<sup>[57]</sup>. Therefore, VEGF

seemed a reasonable target in the treatment of DME and recently, these agents have quickly become the first line of therapy in patients with center-involved DME. There are four currently available anti-VEGF agents.

#### **Pegaptanib (Macugen; OSI pharmaceuticals, Melville, NY)**

Pegaptanib was the first anti-VEGF agent to be approved by food and drug administration (FDA) for intravitreal injection in patients with wet age-related macular degeneration (AMD). A study of pegaptanib in DME showed a better VA at 36 wk following treatment with 0.3 mg pegaptanib as compared to sham. Mean central thickness decreased 68 micron with pegaptanib *vs* an increase of 4 micron with sham ( $P = 0.02$ ). Laser photocoagulation was needed in fewer patients using pegaptanib as compared to sham (25% *vs* 48%). In a recent study of pegaptanib for patients with DME, VA improved  $\geq 10$  letters in 36.8% in the pegaptanib group and 19.7% in the sham group and fewer laser treatments were needed in the pegaptanib group<sup>[58]</sup>.

#### **Ranibizumab (Lucentis; Genentech, San Francisco, CA)**

Ranibizumab (RBZ) is an intravitreal anti-VEGF agent that is FDA approved for the treatment of wet AMD, and has been used for the treatment of DME<sup>[59]</sup>. Several studies have reported the superiority of RBZ as compared to laser treatment. The RESOLVE study<sup>[60]</sup>, a randomized multicenter study, showed that RBZ is more effective in improving vision when compared to sham treatment (with rescue laser) over 12 mo (mean BCVA improvement +10.3 for RBZ *vs* -1.4 for sham,  $P = 0.0001$ ). READ 2 study<sup>[61]</sup> showed that patients who received RBZ alone (group 1) gained an average of 7.4 letters at 6 mo as compared to a 0.5 letter loss in patients receiving macular laser therapy only (group 2) and a 3.8 letters gain in patients received both laser treatment and RBZ (group 3). At 24 mo, and after allowing for RBZ to groups 2 and 3 starting at 6 mo, the mean improvement in BCVA was 7.7, 5.1 and 6.8 letters in groups 1, 2 and 3 respectively<sup>[50]</sup>. The OCT findings, however, did not parallel the visual outcome. The mean foveal thickness at 24 mo was 340  $\mu\text{m}$ , 286  $\mu\text{m}$  and 258  $\mu\text{m}$  for groups 1, 2 and 3 respectively.

The RESTORE study<sup>[62]</sup> was a phase 3 study in Australia that showed that RBZ alone or combined with laser was superior to laser monotherapy in improving BCVA (+6.1 letters for RBZ alone, +5.9 letters for the combination group and +0.8 for the laser group,  $P < 0.0001$ ). The mean central macular thickness was significantly decreased from baseline (-118.7  $\mu\text{m}$ , -128.3  $\mu\text{m}$  and -61.3  $\mu\text{m}$  for RBZ, RBZ + laser and laser alone respectively). Another phase 3 trial from the DRCRnet<sup>[50]</sup> showed that RBZ injection with laser (prompt or deferred) was significantly more effective than laser alone in improving VA in patients with center involved DME after 1 year of treatment (+9 *vs* +3 letters gain;  $P < 0.001$ ). Approximately 30% of the patients gained  $\geq 15$  letters compared to 15% of the laser alone group. Moreover, RBZ was

found to significantly decrease the risk of progression of DR. The RISE and RIDE<sup>[63]</sup> were also phase 3 randomized multicenter studies that addressed the efficacy of RBZ in the treatment of DME. Patients were randomized to 3 groups (sham injection, 0.3 mg RBZ and 0.5 mg RBZ). At 24 mo, 18.1% of the sham group of eyes gained  $\geq 15$  letters *vs* 44.8% of the 0.3 mg and 39.2% of the 0.5 mg treated eyes in the RISE study. Similarly, significantly more RBZ treated patients gained  $\geq 15$  letters in the RIDE study, (12.3% of sham group *vs* 33.6% of the 0.3 mg and 45.7% of the 0.5 mg RBZ patients). RBZ treated eyes underwent significantly fewer macular laser treatments. The above studies confirm that RBZ rapidly and sustainably improves edema and vision, and reduces the risk of further vision loss and progression of DR in patients with DME.

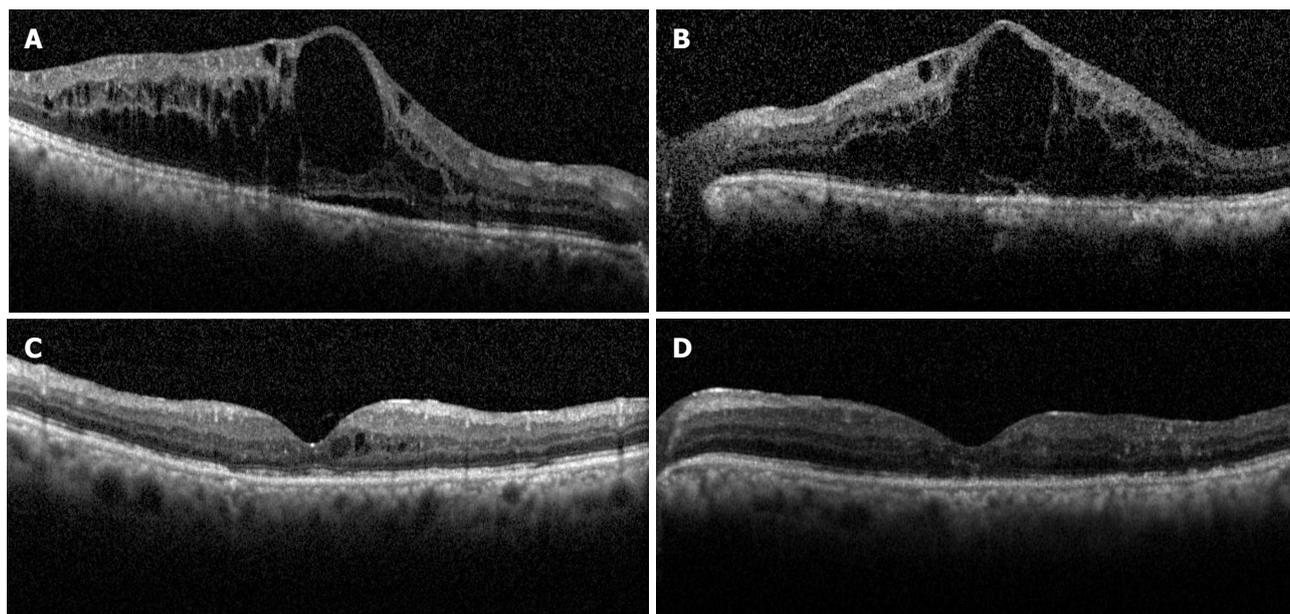
#### **Bevacizumab (Avastin; Genentech, San Francisco, CA)**

Bevacizumab was approved by the FDA for the treatment of colorectal cancer. It has been used off label in the treatment of wet AMD and other ocular diseases including DME<sup>[64]</sup>. Multiple studies showed the beneficial effect of bevacizumab in the treatment of DME<sup>[65-68]</sup> (Figure 2).

In addition, a prospective randomized controlled clinical trial (the BOLT study)<sup>[69]</sup>, found that bevacizumab has a greater treatment effect than macular laser treatment in patients with center involving persistent CSME. At 12 mo, there was a statistical significant difference in the mean ETDRS BCVA ( $P = 0.0006$ ). At 2 years, the mean ETDRS BCVA was 64.4 (ETDRS equivalent Snellen fraction: 20/50) in the bevacizumab group and 54.8 (20/80) in the macular laser therapy group ( $P = 0.005$ ). Also, there was a mean gain of 8.6 letters in the bevacizumab group *vs* a loss of 0.5 letters for the macular laser group. Forty-nine percent of patients gained  $\geq 10$  letters ( $P = 0.001$ ) and 32% gained at least 15 letters ( $P = 0.004$ ) in the bevacizumab group *vs* 7% and 4% in the macular laser group.

#### **Aflibercept (VEGF Trap-Eye, Regeneron Pharmaceutical, Inc, Tarrytown, NY, United States)**

VEGF Trap-Eye (VTE) is the most recent anti-VEGF available for clinical use. Unlike RBZ and bevacizumab, the VTE incorporates the second binding domain of the VEGFR-1 receptor and the third domain of VEGFR-2 receptor to the FC segment of human immunoglobulin G. It has been investigated in the treatment of wet AMD, DME and retinal vein occlusion. The DA VINCI study<sup>[70]</sup>, a randomized multicenter phase 2 clinical trial compared different doses and dosing regimens of VTE with laser photocoagulation in patients with DME. Patients were assigned randomly to 1 of 5 treatment regimens. VTE 0.5 mg every 4 wk, 2 mg ever 4 wk, 2 mg every 8 wk, 2 mg as needed after 3 initial monthly injections or macular laser treatment. The mean improvement in BCVA in the VTE groups at 24 wk ranged from 8.5 to 11.4 letters *vs* 2.5 letters for the laser group. At 52 wk, the mean improve-



**Figure 2** Horizontal spectral-domain optical coherence tomography scans of the macula before (A and B) and after (C and D) intravitreal bevacizumab therapy in a patient with diabetic macular edema. Note extensive cystoid macular edema in both eyes (A and B) and subretinal fluid in the right (A). Six months following treatment with 3 injections of bevacizumab, the macular edema almost completely resolved in both eyes (C and D). The Snellen visual acuity improved from 20/125 to 20/70 in the right eye, but did not change significantly in the left, likely due to atrophic changes in the outer retina as seen on optical coherence tomography.

ment ranged from 9.7 to 12 letters in the VTE groups *vs* -1.3 for laser group. The mean reduction in central retinal thickness in the VTE groups ranged from -165.4 to 227.4 *vs* -58.4 for the laser group. However, phase 3 studies are still pending.

#### Side effects of anti VEGF

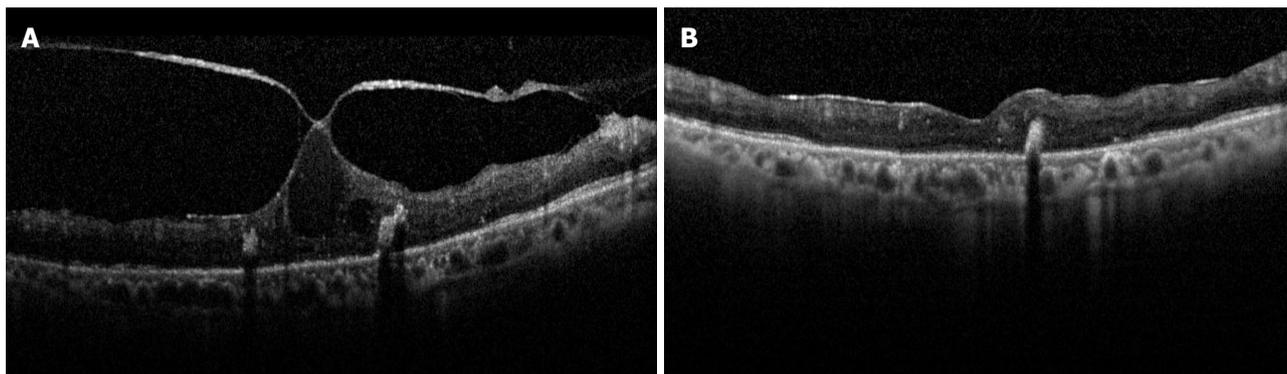
The anti-VEGF agents, particularly RBZ and bevacizumab, are currently widely used in the treatment of center involving DME and are usually well tolerated. There are some potential systemic and ocular side effects that are well recognized. The ocular side effects include inflammation, traumatic injury to the lens, retinal detachment, vitreous hemorrhage and endophthalmitis. Fortunately, these occur at relatively infrequent rates. The potential systemic side effects include cardiovascular and cerebrovascular accidents such as myocardial infarction, pulmonary embolism, hypertension, hemorrhage. Although such systemic side effects have been clearly associated with the systemic use of bevacizumab, their relationship to the intravitreal use of anti-VEGF agents is less well understood, and the data so far suggests that no such cause effect relationship exists<sup>[56]</sup>.

#### VITRECTOMY

The role of the vitreous in the development and progression of DME has been recognized through several mechanical and physiologic mechanisms, all of which are presumed to lead to increased vascular permeability<sup>[71-78]</sup>. The attachment of the posterior hyaloid is a vital observation in eyes with DME which have a lower rate of posterior vitreous detachment than those without<sup>[79]</sup>, and

spontaneous posterior vitreous separation is associated with improvement in DME<sup>[79,80]</sup>. Schepens<sup>[81]</sup> was the first to propose the possible role of vitreous traction in the development of cystoid macular edema associated with retinitis pigmentosa, aphakia, and uveitis. Nasrallah *et al*<sup>[71]</sup> reported the first data to suggest that the vitreous can play a role in the pathogenesis of DME. Among their study population with diabetic retinopathy, DME occurred only in 20% of patients with a posterior vitreous detachment (PVD) compared to 55% in those with an attached hyaloid. Furthermore, Hikichi *et al*<sup>[79]</sup> reported that over a period of 6 mo, DME resolved spontaneously in 55% of the cases with vitreomacular detachment compared to 25% of the cases with vitreomacular adhesion. The efficacy of pars plana vitrectomy (PPV) has been advocated for the treatment of diffuse DME with taut posterior hyaloid. In 1992, Lewis *et al*<sup>[82]</sup> described that PPV was effective for treating DME with thickened and taut posterior hyaloid that did not respond to laser treatment whereby 8 out of 10 patients experience VA and macular thickening improvement. Since then many studies showed that vitrectomy resulted in a decrease of macular edema (Figure 3) and frequently improvement of VA.

Various mechanisms have been assumed to elucidate the beneficial effects of vitrectomy in DME. The vitreomacular traction in both the antero-posterior and tangential dimensions may be important in the pathogenesis of ME which has been well-demonstrated by optical coherence tomography<sup>[83]</sup>. On the other hand, Gandorfer *et al*<sup>[76]</sup>, Ikeda *et al*<sup>[84]</sup> and La Heij *et al*<sup>[85]</sup> showed that vitrectomy was effective even when there is no evidence of macular traction. One possible explanation is that vitrectomy appears to increase oxygenation of the



**Figure 3** Spectral-domain optical coherence tomography scans of the macula before (A) and after (B) pars plana vitrectomy in a patient with diabetic macular edema in the left eye. Note the focal vitreomacular adhesion with associated cystoid macular edema and intraretinal hard exudates. Seven months following surgery, the macular edema completely resolved (B), but the Snellen visual acuity was not changed and measured 20/400 likely due to atrophic changes in the outer retina as seen on optical coherence tomography.

macula. Kadonosono *et al.*<sup>[86]</sup> demonstrated increased peri-foveal capillary blood flow after vitrectomy in their series. Stefansson<sup>[87,88]</sup> reported that vitrectomy with lensectomy allows aqueous to provide increased levels of oxygen to the inner retina and that movement of oxygen trans-corneally to the vitreous has been shown to be more effective in vitrectomized eyes<sup>[89]</sup>. In addition, the removal of the posterior hyaloid also helps in reducing the load of toxic substances and cytokines, such as histamine, free radical scavengers, and VEGF, which may be harbored in the preretinal space in such eyes<sup>[90]</sup>.

Recently, internal limiting membrane (ILM) peeling has been added to vitrectomy for ME though its effect is not yet certain. The ILM in diabetic eyes contains increased levels of fibronectin, laminin and collagen<sup>[91,92]</sup>. Histopathologically, peeled ILM specimens from DME eyes have been shown to be several times thicker than normal ILM, possibly contributing to altered fluid shifts between the vitreous and retina<sup>[93]</sup>. Moreover, Funatsu *et al.*<sup>[94,96]</sup> and Aiello *et al.*<sup>[56]</sup> independently stated a hypothesis that in the vitreous body of diabetic patients there are humoral substances such as VEGF and interleukin 6, generated in the cells of epiretinal membranes, which impair the ocular-vascular barrier and contribute to the pathogenesis of DME. Similarly, Antonetti and associates demonstrated that increased levels of VEGF in the vitreous decrease levels of occludin, a membrane spanning tight junction protein, which could change the structure of the retinal endothelial junction and may account for the increased vasopermeability in patients with DME<sup>[97]</sup>.

The addition of ILM peel to vitrectomy was suggested to yield a better anatomical as well as functional results<sup>[76,98]</sup>, and its effectiveness was sustained long term with less recurrence<sup>[99]</sup>. The mechanism for this was hypothesized to be removal of the scaffold used by astrocytes to proliferate on the retinal surface and the elimination of all tractional forces at the vitreoretinal interface. In addition, it allows the ME to decompress by facilitating the release of extracellular fluid into the vitreous, which restore normal retinal thickness and intraretinal tissue pressure.

Regression of macular edema after PPV and ILM peel may also be explained by a decrease or elimination of factors enhancing vasopermeability. These factors could be produced by cells within the cortical vitreous and ILM, or they could build up in the vitreous and concentrate in the macular region and provoke or aggravate macular edema. This hypothesis is also supported by the gradual postoperation regression of CSME<sup>[100]</sup>.

Surgical removal of the ILM is challenging and can compromise the already affected retinal tissue. Some of the challenges include poor visibility of the thin transparent membrane, the small dimensions and the sensitivity of the macular tissue, as well as the potential staining dye toxicity during the procedure<sup>[101,102]</sup>. In a study by Kumagai *et al.*<sup>[103]</sup> of 135 eyes, 74 of which underwent ILM peeling, although ILM removal accelerated the absorption of ME, the final VA were similar in both groups. Furthermore, Hartley *et al.*<sup>[104]</sup> found that PPV with ILM peeling improves anatomy in DME patients, but does not necessarily improve VA. In one of the DRCR studies, 87 eyes with moderate visual loss which underwent vitrectomy for DME and associated vitreomacular traction were reviewed. As an adjunct to vitrectomy, epiretinal membrane peeling was done in 61%, ILM peeling in 54%, PRP in 40%, and injection of corticosteroids at the end of the procedure in 64%. At 6 mo, a median improvement in VA of 3 letters was reported, with VA improving by  $\geq 10$  letters in 38% (95%CI: 28%-49%) and worsening by  $\geq 10$  letters in 22% (95%CI: 13%-31%). Greater VA improvement was reported in eyes with worse baseline acuity ( $P < 0.001$ ) and in eyes that had epiretinal membrane removal ( $P = 0.006$ )<sup>[105]</sup>. In this study, ILM removal was not one of the factors associated with VA improvement following vitrectomy although it was associated with a better anatomical outcome.

In addition to its controversial role in DME, vitrectomy may be associated with several serious and sight threatening potential complications such as ocular haemorrhage, endophthalmitis, retinal tears and detachment, cataract formation, and glaucoma. Aside from cataract, the rate of these is relatively low fortunately. For ex-

ample, in one study<sup>[106]</sup> post-vitreotomy complications in eyes with DME were as follows: retinal tears (9.6%), epiretinal membrane (9%), retinal detachment, neovascular glaucoma and rubeosis iridis (each 1.9%). Likewise, the DRCRnet study group<sup>[107]</sup> reported postoperative complications at 6 mo as vitreous hemorrhage (5 eyes), elevated intraocular pressure requiring treatment (7 eyes), retinal detachment (3 eyes), and endophthalmitis (1 eye). Few changes in results were observed between 6 mo and 1 year follow up.

## STERIOD IMPLANTS

Chronic diseases, like DME, may be best controlled by treatment modalities capable of providing a therapeutic effect for a prolonged period. Moreover, new formats of drug delivery like long-acting intravitreal drug delivery implants can overcome many of the limitations of other therapeutic approaches, such as frequent intravitreal injections or systemic side effects, by providing long-term localized delivery of therapeutic agents to the posterior segment at sufficiently high local dosages necessary to elicit the desired therapeutic effects.

The Retisert implant [fluocinolone acetonide (FA) intravitreal implant 0.59 mg; Bausch and Lomb, Rochester, NY] is a first-generation device that has been FDA-approved for the treatment of chronic macular edema due to non-infectious uveitis<sup>[108]</sup>. It is composed of a non-biodegradable intravitreal implant that is surgically inserted through a 3.5 mm incision and sutured to the eye wall, where it provides a relatively consistent delivery for about two and a half years. The initial dose is 0.6 µg a day, which decreases over the first month to about 0.3-0.4 µg a day<sup>[109]</sup>. In a multicenter clinical trial Pearson *et al*<sup>[109]</sup> reported a group of 197 patients randomized to receive either sustained release FA intravitreal implant (Retisert) or modified ETDRS laser for DME. Over the 3-year study period, 58% of the eyes in the Retisert group had no evidence of DME, while only 30% of the laser-treated eyes had resolution of the edema ( $P < 0.001$ ). VA improvement of  $> 3$  lines was achieved in 28% of eyes in the Retisert group *vs* 15% in the laser group ( $P < 0.05$ ). Loss of  $> 3$  lines VA was similar in both groups (19% with Retisert *vs* 16% with laser). There were significant adverse effects among the Retisert group which included IOP rise in 35%, the rate of filtration surgery was 28%, and in 5% of eyes the device had to be explanted to manage the IOP. The other major obstacles that restricted the widespread use of the this implant for DME include the need for initial surgical implantation, the potential need to explant empty devices and re-implant loaded ones in the operating room for maintenance of therapy, as well as cost.

Iluvien (Alimera Sciences, Alpharetta, United States), is an injectable second-generation version of the Retisert FA implant that can be delivered in an office setting without surgery using a 25-gauge injector. It can be designed to release either 0.5 µg of FA a day for 18 mo, or 0.2 µg

a day for three years. A pharmacokinetic study revealed that each supplied excellent sustained delivery of FA in the eye for at least 1 year and reduced DME<sup>[111]</sup>.

Based on the clinical results and safety profile perceived in the Retisert studies, the Iluvien insert has entered phase 3 testing in DME. The Fluocinolone Acetonide in Diabetic Macular Edema (FAME) study was a randomized, double-masked, multicenter clinical trial that compares the 0.5 µg/d and 0.2 µg/d injectable Iluvien inserts with sham injection among patients with persistent DME despite focal laser. Over 24 mo of follow-up, 28.7% and 28.6% in the low- and high-dose insert groups, respectively, reported an improvement in ETDRS vision of 15 or more letters compared with 16.2% in the sham group ( $P = 0.002$  for each). The mean improvement in BCVA letter score between baseline and month 24 was 4.4 and 5.4 in the low- and high-dose groups, respectively, compared with 1.7 in the sham group ( $P = 0.02$  and  $P = 0.016$ ). Subjects needing cataract surgery were more frequent in the insert groups, and their visual benefit was similar to that of subjects who were pseudophakic at baseline. Glaucoma requiring incisional surgery reported in 3.7%, 7.6% and 0.5% of the low-dose, high-dose and sham groups, respectively<sup>[112]</sup>.

Another device being studied in the setting of DME is Ozurdex (dexamethasone intravitreal implant), a biodegradable implant that can be placed in the vitreous cavity by a 22-gauge applicator through a small self-sealing puncture. The implant may contain either 350 or 700 mcg of dexamethasone and the drug is released in a biphasic fashion, with very high doses for up to six weeks, followed by low but therapeutic doses out to six months or more. In a randomized, controlled clinical trial to evaluate the safety and efficacy of dexamethasone intravitreal drug delivery system (DDS) among patients with persistent DME, patients were randomized to treatment with 700 mcg or 350 mcg of DDS or observation. At day 90, more eyes in the 700 (33.3%) and 350 (21.1%) mcg groups reported a BCVA improvement of 10 letters or more compared with the observation group (12.3%;  $P = 0.007$  *vs* 700-mcg group). The treatment benefit was less noticeable at 180 d where 30% of eyes in the 700 mcg group, 19% in the 350 mcg group, and 23% in the observation group experienced a BCVA improvement of 10 letters or more ( $P \geq 0.4$  for treated *vs* observed eyes). Moreover, a statistically significant reduction in both OCT retinal thickness and leakage by fluorescein angiography was also demonstrated in both implanted dosages *vs* controls ( $P = 0.03$ ; day 90)<sup>[113]</sup>.

A prospective, multicenter, open-label, 26-wk study was designed to evaluate the safety and efficacy of Ozurdex 0.7 mg received in a single dose by 55 patients with treatment-resistant DME (mean duration of 43 mo) and a history of previous PPV. At week 8, the mean change from baseline central retinal thickness (403 µm) was -156 µm ( $P < 0.001$ ) and -39 µm at week 26 ( $P = 0.004$ ). The mean increase in BCVA from baseline (54.5 letters) was 6.0 letters at week 8 ( $P < 0.001$ ) and 3.0 letters at week 26

( $P = 0.046$ ). At week 8, a gain of  $\geq 10$  letters in BCVA were reported in 30.4% of patients<sup>[114]</sup>.

A number of other studies evaluating the Ozurdex implant for the treatment of DME as monotherapy, in combination with laser, and in post-vitreotomized eyes have been carried out as well. The results of the pivotal phase 3 trial (MEAD), comparing two doses of the dexamethasone implant with sham treatment, are not yet available, although recruitment is complete, and full three-year follow-up is expected to be complete by the end of 2012<sup>[115]</sup>. Another phase 3 trial, PLACID, compared the safety and efficacy of the dexamethasone implant plus laser with sham implant procedure plus laser in the treatment of DME. This one-year trial (results reported at scientific conferences, not yet published) which permitted reinjection of Ozurdex after six months, showed that patients with diffuse DME had better BCVA at 12 mo when treated with Ozurdex plus laser vs laser alone<sup>[116]</sup>. The trial in postvitreotomized eyes with persistent DME (the CHAMPLAIN study) was a 26-wk open-label single Ozurdex injection trial. The study reported that 30% of eyes had experienced a two-line improvement in BCVA by 13 wk, although this effect diminished by the study endpoint of 26 wk<sup>[114]</sup>.

In terms of adverse events, the dexamethasone implant was well tolerated and most events were mild in severity. High IOP was successfully managed with either observation or topical medications; none required laser or surgical intervention to control the IOP. There was no statistically significant difference between groups in the occurrence of any of the non-ocular adverse events<sup>[113]</sup>.

## MISCELLANEOUS

### Micropulse Laser

The value of conventional laser photocoagulation for DME was well established since the ETDRS reported its results and the procedure involves production of visible burns in the retina, which when observed, indicate that the thermal spread has reached the overlying neurosensory retina with a temperature high enough to alter its natural transparency. Enlargement of laser scars after treatment has been reported<sup>[23]</sup>. A sub-threshold micropulse diode laser (with 810 nm and 577 nm lasers), seems to have a theoretical advantage since the laser burns will selectively affect deeper layers with relative sparing of the inner neurosensory retina, thus reducing the scarring and paracentral scotomas post-treatment<sup>[15]</sup>.

Using a micropulse mode, laser energy is delivered with a train of repetitive short pulses (typically 100 to 300 microseconds “on” and 1700 to 1900 microseconds “off”) within an “envelope” whose width is typically 200 to 300 milliseconds<sup>[117]</sup>. Micropulse power as low as 10% to 25% of the visible threshold power has been revealed to be sufficient to show consistent RPE-confined photo thermal effect with sparing of the neurosensory retina on light and electron microscopy<sup>[118]</sup>.

Micropulse has been demonstrated to be as effec-

tive as conventional argon laser for DME by several authors<sup>[15,119-122]</sup>. In order to assess the efficacy of subthreshold micropulse diode laser photocoagulation for DME, a prospective, nonrandomized interventional case study was carried out evaluating forty-three eyes (36 patients) with CSME and a central macular thickness (CMT) of  $< 600$  micron on OCT. A significant reduction of CMT was detected within the first 3 mo of the laser treatment, but the changes of BCVA and macular volume were not significant. After 12 mo, 94.7% of the patients had a VA that was either improved or preserved within 0.2 logMAR. Laser scars were not identified in any patient in this study<sup>[121]</sup>.

In another prospective, randomized study using multifocal electroretinography (MfERG) the efficacy of subthreshold micropulse diode laser was compared against double-frequency neodymium YAG (Nd:YAG) laser. Thirty three patients (46 eyes) with CSME were randomized to either SDM (810 nm) laser or the conventional double-frequency Nd:YAG (532 nm) laser. At 6 mo, no statistically significant differences were reported for all of the following: CMT as measured by OCT and macular retinal sensitivity measured using MfERG (as a primary outcome measures), BCVA and contrast sensitivity (as secondary outcome measures), between the two groups. The authors concluded that subthreshold micropulse diode laser photocoagulation showed an equally good effect on VA, contrast sensitivity, and DME as compared to conventional Nd:YAG laser photocoagulation with a potentially better preservation of retinal tissue and electrophysiological indices<sup>[122]</sup>.

In the anti-VEGF era, subthreshold tissue-sparing therapy may play a major role in the management of DME in the future, especially when considering combining it with intravitreal injections. This regimen may prove helpful in reducing the number of injections needed to control the edema. However, future studies are needed in this regard.

### Anti-tumor necrosis factor alpha (or tumor necrosis factor antagonist)

Several levels of evidence suggest an inflammatory basis for DME<sup>[123]</sup>. Moreover, many authors reported data supporting the important role of an altered local expression of TNF in the pathogenesis of DME<sup>[124,125]</sup> and that low-grade subclinical inflammation is responsible for many of the characteristic vascular lesions of diabetic retinopathy<sup>[123]</sup>. Along this line, treatment modalities have been tried with variable success.

The monoclonal anti-TNF antibody Infliximab neutralizes TNF actions and has been used for inflammatory arthritic conditions and Crohn's disease since 1998 with a favorable safety profile<sup>[126]</sup>. Studies in patients with arthritis have shown that anti-TNF therapy negatively affects vascular permeability and angiogenesis by decreasing VEGF<sup>[127]</sup>, which has been implicated directly in the pathogenesis of DME and diabetic retinopathy<sup>[161,128]</sup>. Likewise, repeated treatment in one diabetic patient

produced a further significant improvement of DME, suggesting that the clinical response to anti-TNF dosing regimens is individualized, as observed in patients with arthritis, or in patients with uveitic macular edema<sup>[126]</sup>.

The efficacy and safety of infliximab in treating patients at risk of vision loss secondary to DME refractory to laser photocoagulation was evaluated by a small phase III prospective, double-blind, randomized, placebo-controlled study. At weeks 0, 2, 6 and 14, eleven patients received infliximab (5 mg/kg) intravenously, followed by placebo at weeks 16, 18, 22 and 30, or vice versa. VA improved from 23.5 + 10.3 (mean + SD letters read) at baseline to 30.4 + 13.4 letters at week 16 in 8 infliximab-treated eyes that were eligible for analysis, and the effect was sustained till the completion of placebo treatment (31.4 + 12.1). A higher proportion of eyes experienced VA improvement in the infliximab-treated eyes ( $P = 0.017$ ). Infliximab treatment was well tolerated in this study<sup>[129]</sup>.

Further studies examined the use of intravitreal TNF inhibitors but the results were not encouraging. An interventional, retrospective, multicenter study by Wu and associates, evaluated the short-term visual and anatomical outcomes after intravitreal injections of two TNF- $\alpha$  inhibitors (adalimumab 2 mg or infliximab 1 mg or 2 mg) in 39 eyes with refractory DME. No apparent benefit for treatment was reported in any of the study groups. Additionally, no ocular side effects were documented in the eyes injected with either adalimumab or 1 mg of infliximab, while severe uveitis was reported in 42% (8 of 19) of the eyes receiving 2-mg infliximab. All were controlled with topical steroid therapy except three eyes (37.5%) required PPV<sup>[130]</sup>. Similarly, in a prospective, noncomparative, interventional small case series of four patients, with age-related macular degeneration, half of the cases developed intraocular inflammation with high intraocular pressure 3 and 5 wk after the infliximab injection, respectively. One case was controlled with topical medication, and one case required posterior vitrectomy<sup>[131]</sup>. Tsilimbaris *et al.*<sup>[132]</sup>, in a pilot study of seven patients with refractory DME, reported no definite benefit to intravitreal etanercept after 2 consecutive intravitreal injections of 2.5 mg (0.1 mL) at a two-week interval. Three months after initiation of therapy, analysis of the data shows a tendency for improvement of VA, a slight worsening of hard exudates and fluorescein leakage, while hemorrhages remained stable, however, no statistical significance for therapy was attained. No adverse reactions or adverse events were observed in any patient. Therefore, the role of TNF inhibition in patients with DME remains to be determined.

#### **PKC beta-isoform inhibitors (or Ruboxistaurin)**

Substantial data suggest that the Protein Kinase-C  $\beta$  (PKC- $\beta$ ) may play an important function in regulating endothelial cell permeability and is an important triggering component for VEGF<sup>[132]</sup>. Inhibition of PKC  $\beta$ -isoform activity was shown to reduce diabetes-induced retinal permeability and ischemia-induced retinal neovasculariza-

tion<sup>[133,134]</sup>. Zhu *et al.*<sup>[135]</sup> also reported that the enhanced endothelin-1 (ET-1) expression associated with the activation of PKC occurs in early diabetes, and PKC inhibitor could reverse the up regulation of ET-1.

Ruboxistaurin (RBX), an orally active PKC- $\beta$  inhibitor, has revealed inconsistent results against DME. In several animal models, RBX improved hyperglycemia-induced diabetic microvascular complications, including diabetic retinopathy and DME<sup>[133]</sup>. The PKC- $\beta$  Inhibitor-Diabetic Retinopathy study 2 (PKC-DRS2)<sup>[136]</sup>, documented that treatment with oral RBX (32 mg/d) decreased moderate visual loss [9.1% of placebo-treated patients *vs* 5.5% of RBX-treated patients (40% risk reduction,  $P = 0.034$ )], macular edema progression (68% *vs* 50%,  $P = 0.003$ ), and the need for laser treatment for macular edema (26% less frequent in eyes of RBX-treated patients,  $P = 0.008$ ), with increasing occurrence of visual improvement in patients with nonproliferative retinopathy. Although RBX was well tolerated and reduced the risk of visual loss, it did not prevent DR progression. On the other hand, the protein kinase C-Beta inhibitor Diabetic Macular Edema study (PKC-DMES)<sup>[137]</sup> reported some evidence that ruboxistaurin was associated with reduced progression of DME, although this was a secondary endpoint. Ruboxistaurin has not received approval from the United States FDA.

#### **Nicotinic acetylcholine receptor antagonist (Mecamylamine)**

In the 1950s, mecamylamine [a nonspecific nicotinic acetylcholine (nACh) receptor antagonist] was approved as an oral antihypertensive agent<sup>[138]</sup>. In animal models, it could inhibit angiogenesis and vascular permeability by blocking nACh receptors on vascular endothelial cells. Its application using a topical ocular formulation containing 0.1% or 1% mecamylamine 3 times daily in rabbits resulted in detectable levels of mecamylamine in the retina and suppressed CNV in mice<sup>[139]</sup>. A multicenter phase I / II clinical trial reviewed the safety and bioactivity of topical mecamylamine in twenty-three patients with chronic DME. Over a period of 12 wk, all eyes were treated twice daily with topical 1% mecamylamine. The mean improvement in BCVA was 2.8, 1.9, 2.4, 0.8 and 3.1 letters at 1, 4, 8, 12 and 16 wk respectively, with little associated change in mean excess foveal thickness. There were no recognized drug-related safety problems<sup>[140]</sup>.

Although these findings are encouraging especially for a topical formulation, further studies are needed to address the efficacy of the medication especially in relation to other available therapies such as the intravitreal anti-VEGF agents.

#### **Pharmacologic vitreolysis (Microplasmin-ThromboGenics)**

As discussed above in the section on "vitrectomy", vitreous traction is one of the causes for persistence of DME in some cases. Inducing a PVD could be one line of management for these cases. Although the surgical vit-

rectomy instrumentation and techniques have markedly improved in recent years, surgery is still associated with several potential complications<sup>[141,142]</sup>. As a result, it would be useful to have a biochemical agent that could cleave the vitreoretinal interface selectively with a relatively good safety profile.

Recently, pharmacologic vitreolysis has been explored as a new modality of treatment to manage DME. Microplasmin, which is approved by the United States-FDA, has shown promise in inducing a posterior vitreous detachment when given as a single intravitreal injection. Microplasmin is a truncated form of plasmin, an enzyme that dissolves protein formations that are crucial to blood clot (thrombus) formation; similar protein formations are seen linking the vitreous to the retina in the eye. It has the benefit of reaching a peak of action after 15-30 min of administration and continuing in a plateau for another 90 min then decreases to undetectable levels in 24 h<sup>[143]</sup>. A phase 2, multicenter, placebo-controlled, double-masked, clinical trial was designed to evaluate the safety and efficacy of a preoperative intravitreal injection of microplasmin in patients ( $n = 125$ ) scheduled for vitreous surgery. The rate of total PVD at the time of surgery were reported in 10%, 14%, 18%, and 31% in the placebo group, 25-microg, 75-microg and 125-microg microplasmin groups, respectively. The secondary end point of resolution of vitreomacular interface abnormality precluding the need for vitrectomy at the 35-d time point was observed at the rates of 3%, 10%, 15% and 31% respectively. However, a more definitive evaluation of the efficacy of microplasmin in the management of patients with DME in phase 3 clinical trials is warranted<sup>[144]</sup>.

## OTHERS THERAPIES

A range of new additional medical and surgical therapies and investigational modalities show potential for the management of refractory DME. Nepafenac, the amide analog of the NSAID amfenac, is hydrolyzed by the uveal tissue and retina<sup>[145]</sup> where it could probably reduce vascular permeability by inhibiting the inflammatory cascade. A small case series<sup>[146]</sup> suggested that topical nepafenac might have a beneficial effect in the treatment of DME.

Another example is Sirolimus that has been documented to inhibit the production and activity of many growth factors related to the development of diabetic retinopathy. In a phase 2 clinical study<sup>[147]</sup>, it was shown that repeated subconjunctival sirolimus injections were well-tolerated with no significant drug-related undesirable events. There was an inconsistent treatment effect related to sirolimus, which deserved further assessment by a randomized trial to reveal any possibility of treatment benefits.

In advanced cases of extensive lipid maculopathy where other treatments are usually ineffective and where an increased risk of subretinal fibrosis exists, surgical removal of massive deposition of macular hard exudates was tried by several retina surgeons<sup>[148]</sup>. Takagi *et al.*<sup>[149]</sup>,

Sakuraba *et al.*<sup>[150]</sup>, and Takaya *et al.*<sup>[151]</sup> have removed macular hard exudates surgically following vitrectomy. Despite good anatomical results, sustained VA improvement was not observed because of atrophic or degenerative changes. At present, more experience is required to understand the long-term benefit and timing of such a modality of treatment.

Lastly, the new ultra-widefield fluorescein angiography (capturing an image up to 200 degrees) offers a significant tool for assessment of the macula as well as the retinal periphery, and may yield additional information as to any potential role for the peripheral retina in the pathogenesis of DME. Freyler *et al.*<sup>[152]</sup>, suggested a likely association between DME and peripheral non-perfusion after a study with a 60-degree wide-angle angiography camera. In 2005, Kimble *et al.*<sup>[153]</sup> reported that peripheral capillary nonperfusion was detected in 84% of patients with CSME and nonproliferative retinopathy. The RaScal (Ranibizumab + Scatter Laser) study, a small pilot trial, hypothesized that DME is driven predominantly by peripheral retinal ischemia. The patients were randomized to either conventional treatment (macular laser plus IVTA) or a novel treatment strategy of ultra-widefield fluorescein angiography-directed peripheral laser plus intravitreal RBZ. This study revealed a higher recurrence of DME needing retreatment in macular laser + steroid (4/5) *vs* RaScal (1/5), and improvement in central foveal thickness in both groups which was better in RaScal at 6 mo. These findings need to be confirmed by larger studies. In addition the differential effect of ranibizumab *vs* peripheral scatter laser on DME needs to be addressed<sup>[154]</sup>.

In summary, although laser photocoagulation has been the gold standard of therapy for DME, it is being quickly replaced by intravitreal pharmacotherapy as evidenced by three phase 3 clinical trials supporting the superiority of ranibizumab for center involved edema. The role of other agents such as intravitreal triamcinolone injections, steroid implants and aflibercept, as well as that of vitrectomy remains to be determined. While the use of intravitreal triamcinolone and vitrectomy appear to be currently limited to cases that have failed other available therapeutic options, the former may be effective in pseudophakic patients who have no personal or family history of glaucoma, and the latter in cases with evidence of vitreomacular traction. Several new treatment modalities for DME are in the horizon of which topical therapy may play a major role in the future with all the advantages the topical route may offer compared to the intravitreal route.

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## Changes of phasic and tonic smooth muscle function of jejunum in type 2 diabetic Goto-Kakizaki rats

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### Abstract

**AIM:** To generate phasic and tonic stress-strain curves for evaluation of intestinal smooth muscle function in type 2 diabetic rats during active and passive conditions.

**METHODS:** Seven diabetic Goto-Kakizaki (GK) male rats, 32-wk old (GK group), and 9 age-matched normal Wistar rats (Normal group) were included in the study. Jejunal segments were distended up to a pressure of 10 cm H<sub>2</sub>O in an organ bath containing 37 °C Krebs solution with addition of carbachol (CA). The pressure and outer diameter changes were synchronously recorded. Passive conditions were obtained using calcium-free Krebs solution containing ethylene glycol tetraacetic acid and papaverine. Total phasic, tonic and passive circumferential stress and strain were computed from the diameter and pressure data with reference

to the zero-stress state geometry. The active phasic and tonic stresses were defined as the total phasic and tonic stresses minus the passive stress.

**RESULTS:** Diabetes increased jejunal mucosa and muscle layer thicknesses compared to the Normal group (mucosa, 755.8 ± 63.3 vs 633.1 ± 59.1 μm,  $P < 0.01$ ; muscle, 106.3 ± 12.9 vs 85.2 ± 11.7 μm,  $P < 0.05$ ). The pressure and stress thresholds were decreased in the GK group after CA application compared to distensions without CA application (pressure, 1.01 ± 0.07 vs 1.99 ± 0.19 cmH<sub>2</sub>O,  $P < 0.01$ ; stress, 0.11 ± 0.01 vs 0.24 ± 0.02 kPa,  $P < 0.01$ ). CA application did not change the pressure and stress threshold in the Normal group (pressure, 2.13 ± 0.32 vs 2.34 ± 0.32 cm H<sub>2</sub>O,  $P > 0.05$ ; stress, 0.25 ± 0.03 vs 0.35 ± 0.06 kPa,  $P > 0.05$ ). The amplitude of total phasic, total tonic, active phasic and active tonic circumferential stresses did not differ for the distensions without CA application between the GK group and the Normal group. However, the total phasic and total tonic stresses increased after CA application in the GK group compared those in the Normal group. When normalized to muscle layer thickness, the amplitude of active stresses before CA application was lowest in the GK group compared with the Normal group. No difference was found during CA application.

**CONCLUSION:** The stress generated by intestinal muscle normalized to the muscle layer thickness was lowest in GK rats compared to normal rats whereas the response to CA stimulation was preserved.

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**Key words:** Intestine; Diabetes; Muscle function; Stress-strain curves; Carbachol; Rat

**Core tip:** Length-tension diagrams of smooth muscle strips were obtained in intact segment of intestine *in*

*in vitro* in the present study. We demonstrated that it is a valid tool to evaluate smooth muscle function in intact intestine. Diabetes decreased the force (stress) generated by the smooth muscle normalized to the muscle layer thickness. Since the stress decreased and the muscle layer thickness increased in diabetic rats, it indicates that the intestine, at least in part, remodels in a stress-dependent way. Furthermore, the smooth muscle in Goto-Kakizaki diabetic intestine preserved its response to carbachol stimulation.

Zhao JB, Chen PM, Gregersen H. Changes of phasic and tonic smooth muscle function of jejunum in type 2 diabetic Goto-Kakizaki rats. *World J Diabetes* 2013; 4(6): 339-348 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/339.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.339>

## INTRODUCTION

Gastrointestinal (GI) disorders are quite common in diabetic patients<sup>[1]</sup>. Diabetes can affect the entire GI tract including the small intestine. Intestinal motility disorders are common in diabetic patients<sup>[1]</sup> and diabetic animals<sup>[2]</sup>. Delayed as well as rapid small intestinal transit was observed in diabetic animal models<sup>[3-6]</sup>. Small intestinal transit disorders have also been demonstrated in diabetic patients<sup>[7-13]</sup>. Camilleri and Malagelada<sup>[14]</sup> reported that intestinal motility was abnormal in about 80% patients with long-standing diabetes with delayed gastric emptying. Both postprandial and fasting intestinal motility disorders were reported in diabetic patients<sup>[15,16]</sup>.

The pathogenesis of intestinal motility disorders in diabetes is complex in nature, multi-factorial (smooth muscle dysfunction, mechano-sensory disorders, autonomic neuropathy, lack of glycemic control, *etc.*) and is not well understood<sup>[1]</sup>. Our previous studies have shown that the morphological and biomechanical properties of GI tract are altered in type-1 diabetic patients<sup>[17]</sup> and animals<sup>[18-20]</sup>. Similar remodeling was also demonstrated in the esophagus<sup>[21]</sup>, stomach<sup>[22]</sup> and intestine<sup>[23]</sup> in type-2 diabetic animals. Intestinal morphometric and biomechanical remodeling caused by diabetes may change the smooth muscle function, resulting in altered intestinal motility<sup>[20]</sup>. However, to the best of our knowledge, contraction thresholds and muscle mechanical properties described in terms of stress and strain in the diabetic intestine have not yet been reported.

It is possible to obtain isometric length-tension diagrams of phasic and tonic smooth muscle contractions *in vitro*<sup>[24]</sup>. Tools have now been developed for studying the active (phasic and tonic contractions) and passive length-tension behavior in the human gut *in vivo* using impedance planimetric distension<sup>[25-27]</sup>. From a biomechanical standpoint, muscle mechanical properties must be described in terms of stress and strain, *i.e.*, the force per area and tissue deformation. Computation of the stress depends on the wall thickness which can not

be directly measured *in vivo*. However, it is possible to measure the wall thickness *in vitro* and thus to obtain the stress-strain relationship of the intestinal wall with reference to the zero-stress state<sup>[28,29]</sup>. Hence tonic and phasic stress-strain curves of intestinal contractions can be obtained and by using calcium-free solution containing ethylene glycol tetraacetic acid (EGTA) and papaverine to abolish smooth muscle contractions the passive properties can be obtained as well<sup>[28,29]</sup>.

Carbachol (CA) is a parasympathomimetic drug that directly stimulates cholinergic receptors<sup>[30]</sup>. It may also act indirectly by promoting release of acetylcholine and by a weak anticholinesterase action<sup>[31]</sup>. In the present study the intestinal contraction thresholds and the stress-strain curves of smooth muscle contraction were also investigated in response to CA application.

The hypothesis was that the intestinal contraction thresholds and muscle contraction stress-strain curves are altered due to the tissue remodeling induced by diabetes. The aim of this study was to compute stress-strain data for assessment of intestinal smooth muscle function in normal and Goto-Kakizaki (GK) diabetic rats. The contraction threshold and the contraction stress-strain curve in normal and GK diabetic rats during and without CA application are compared.

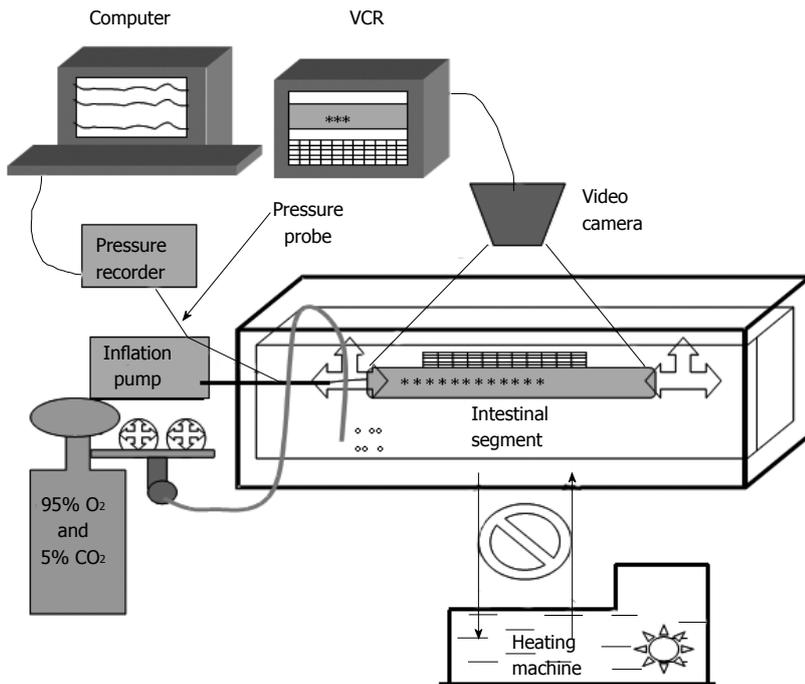
## MATERIALS AND METHODS

### Animals and groups

Seven inherited type 2 diabetic rats (Goto-Kakizaki rats, GK group, 12-wk old, weighing 330 g) were purchased from Taconic Europe DK-8680 Ry, Denmark. Nine age-matched normal rats (same strain as the GK rats) served as controls (Normal group). The rats lived for 32 wk with free access to tap water and food. The body weight was measured on a weekly basis from birth. The fasting glucose level was measured every second weeks from 16 wk after birth and at the day where the experiment was done. Approval of the protocol was obtained from the Danish Committee for Animal Experimentation.

### *In vitro* intestinal preparation

When the scheduled time had arrived, the rats were fasted overnight and then anesthetized with Hypnorm 0.5 mg and Dormicum 0.25 mg per 100 g body weight. The abdominal cavity was opened and a 10-cm-long jejunal segment from 5 cm distal to the ligament of Treitz was harvested. The residual contents in the lumen were gently cleared using physiological saline. One-cm-long specimens were cut from each end of the segment and fixed in 10% formalin for histological examination. Two tissue rings from the proximal end of the segment were cut and used for no-load state and zero-stress state analysis. The remaining segment was inserted into the organ bath containing Krebs solution of the following composition (mmol/L): NaCl, 118; KCl, 4.7; NaHCO<sub>3</sub>, 25; NaH<sub>2</sub>PO<sub>4</sub>, 1.0; MgCl<sub>2</sub>, 1.2; CaCl<sub>2</sub>-H<sub>2</sub>O, 2.5; glucose, 11; ascorbic acid, 0.11 as soon as possible. The Krebs solution was 37 °C aerated with a gas mixture (95% O<sub>2</sub> and 5% CO<sub>2</sub>,



**Figure 1 Experimental set-up.** The organ bath is composed of an inside chamber and an outside chamber. The Krebs solution contained in the small chamber was maintained constant at 37 °C by circulating hot water in the big chamber using a heating machine. The intestinal segment was placed in the small organ bath in the Krebs solution. The intestinal distension was applied by a pump and a pressure probe was used to measure the pressures. The diameter changes of the intestinal segments were videotaped through a stereomicroscope.

pH 7.4). Thirty minutes equilibrating time was needed for recovery of the motility.

**Ramp distension experimental set-up**

The experimental set-up is shown in Figure 1. The proximal and distal ends of each jejunal segment were tied with silk threads onto two cannulas fixed on two sides inside the organ bath. The resting length of the segment (as measured *in vivo* before excising the segment) was adjusted between two cannulas. The proximal cannula was connected *via* a tube to a fluid container containing the same Krebs solution as mention above. Ramp distension were done using a pump (Genie Programmable Syringe Pump, World Precision Instrument, Stevenage, United Kingdom). After two preconditioning cycles (inflation from 0 to 10 cm H<sub>2</sub>O and back again), a ramp distension (0-10 cm H<sub>2</sub>O) was done with closed outlet. The intestinal diameters corresponding to pressure recordings in the intestinal segments were videotaped by a CCD camera (Sony, Japan) through a stereomicroscope during the distensions. The pressure and diameter data acquisition was done at 10 frames per second.

Three minutes after finishing the experiment above, the cholinergic agonist CA (final concentration: 10<sup>-5</sup> mol) was applied to the organ bath. The same procedure as described above was repeated. Then the Krebs solution was replaced by calcium-free Krebs solution containing 0.4% EGTA and 2 mg papaverine in order to abolish smooth muscle contractions. The same protocol as described above was repeated again. The papaverine causes elevation of cyclic adenosine monophosphate levels; alter mitochondrial respiration and inhibit calcium influx by inhibiting enzyme phosphodiesterase.

**Zero-stress state of the intestinal segment**

The method for determination of the intestinal zero-

stress state has been described in detail previously<sup>[19,32]</sup>. One-two millimetre wide intestinal rings were transferred to calcium-free Krebs solution containing EGTA and papaverine. A photograph was taken of the cross-section of the rings in the no-load state. Then, each ring was cut radially under the microscope resulting in an open sector geometry. Photographs of the zero-stress state were taken approximately 60 min after the radial cutting to allow viscoelastic creep to occur.

**Histological analysis of the small intestine**

The intestinal segment was fixed in 10% buffered formalin over 24 h followed by dehydration in a series of graded ethanol (70%, 96% and 99%) and embedding in paraffin. Five-micron sections were cut perpendicular to the mucosa surface and the paraffin was cleared from the slides with coconut oil (over 15 min, 60 °C). Redehydration was done in 99%, 96% and 70% ethanol followed by staining with hematoxylin and eosin. The layer thickness was measured by the same pathologist in a blinded review.

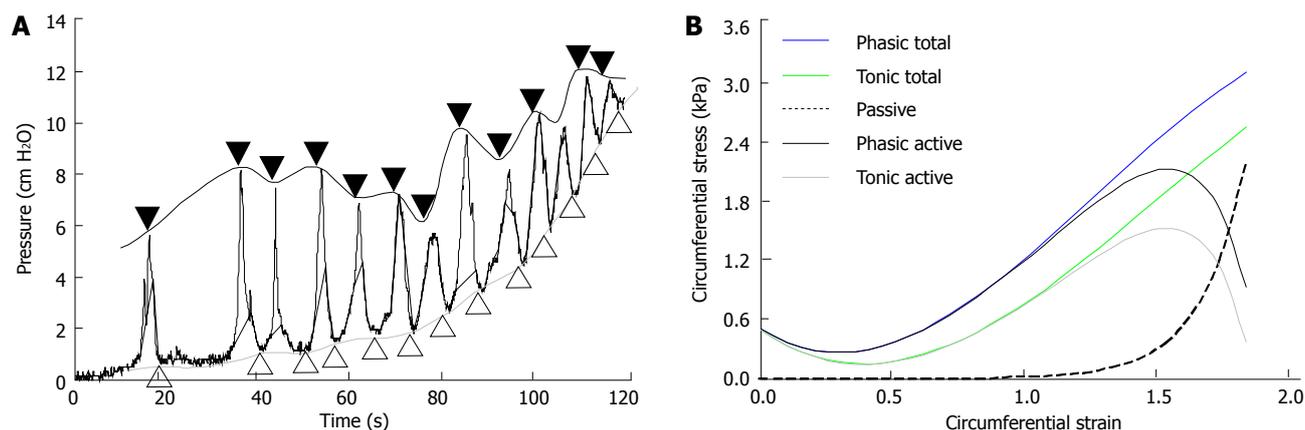
**Mechanical data analysis**

Calculation of mechanical parameters was based on the assessment of the no-load state, zero-stress state dimensions and the outer diameters of the specimen at varying pressures<sup>[32]</sup>. The Kirchhoff's stress and Green's strain in the intestinal wall at a given pressure were computed assuming circular geometry as:

$$\text{Circumferential Kirchhoff's stress: } S_{\theta} = \frac{\Delta P r_{r-p}}{h_p \lambda_{\theta}^2} \quad (1)$$

$$\text{Circumferential midwall Green's strain: } E_{\theta} = \frac{\lambda_{\theta}^2 - 1}{2} \quad (2)$$

where  $\Delta P$  is the transmural pressure difference,  $r$  is the luminal radius;  $h$  is the wall thickness; and  $\lambda_{\theta}$  is the cir-



**Figure 2** Pressure-diameter curve used for set-up maximum and baseline of contraction, and example of stress-strain curves. A: Maximum amplitudes during contraction and the baseline between the contractions during distension. The closed symbols above the curve mark the phasic part (—). The open symbols under the curve mark the tonic part (—). The pressures from the phasic and tonic parts were used to compute the total phasic and tonic stresses; B: An example of passive, total phasic, total tonic, phasic active and tonic active stresses as function of strains from a normal animal. The passive stress increased exponentially as function of strain, whereas the total phasic and tonic stresses increased in a polynomial way. The phasic and tonic active stresses were obtained by the total phasic and tonic stresses minus the passive stress.

cumferential stretch ratio. Pressure, stress and strain data exactly when the contraction started were used to analyze the contraction thresholds.

The total phasic and the total tonic stresses (both composed of active and passive tissue properties) were extracted from the top points (maximum pressure during the contraction) and the baseline pressure between contractions during the distension (Figure 2A). The passive stress was extracted from the data obtained during distension in the calcium-free Krebs solution with EGTA and papaverine. Since strain was not controlled, it was not possible to directly compare strain between different samples and groups. Therefore, curve fitting was applied and strain points were selected for comparison between samples and groups.

The passive stresses increased in an exponential-like way as function of strain. Consequently the passive stress-strain curves were fitted to the exponential function equation

$$S = (S^* + b) e^{a(E - E^*)} - b \tag{3}$$

where  $S^*$  and  $E^*$  are the stress and strain at a physiological reference level. The total phasic and tonic stress-strain curves increased in a polynomial way as function of strain. Consequently the stress-strain curves were fitted to the polynomial equation

$$S = S_0 + a_1 E^3 + a_2 E^2 + a_3 E^1 \tag{4}$$

where  $a_1$ ,  $a_2$  and  $a_3$  are constants. Total stresses and active stresses (see below) were calculated both for distensions before and during CA application. The active phasic and tonic stresses were defined as the total phasic and tonic stresses minus the passive stress (Figure 2B).

$$\text{Active phasic stress} = \text{total phasic stress} - \text{passive stress} \tag{5}$$

$$\text{Active tonic stress} = \text{total tonic stress} - \text{passive stress} \tag{6}$$

The active phasic and tonic stresses were normalized to muscle layer thickness as:

$$\text{Normalized active phasic stress} = \frac{\text{active phasic stress}}{\text{muscle thickness}} \tag{7}$$

$$\text{Normalized active tonic stress} = \frac{\text{active tonic stress}}{\text{muscle thickness}} \tag{8}$$

The muscle layer thickness ( $\mu\text{m}$ ) was obtained from histological measurement.

### Statistical analysis

The results were expressed as mean  $\pm$  SEM unless indicated otherwise. The total phasic, total tonic, active phasic and active tonic stresses were compared between the GK and the Normal groups by analysis of variance and *t* test analyses. The normalized active phasic and tonic stresses as function of strain were also compared between the GK and the Normal groups. The results were regarded as significant when  $P < 0.05$ .

## RESULTS

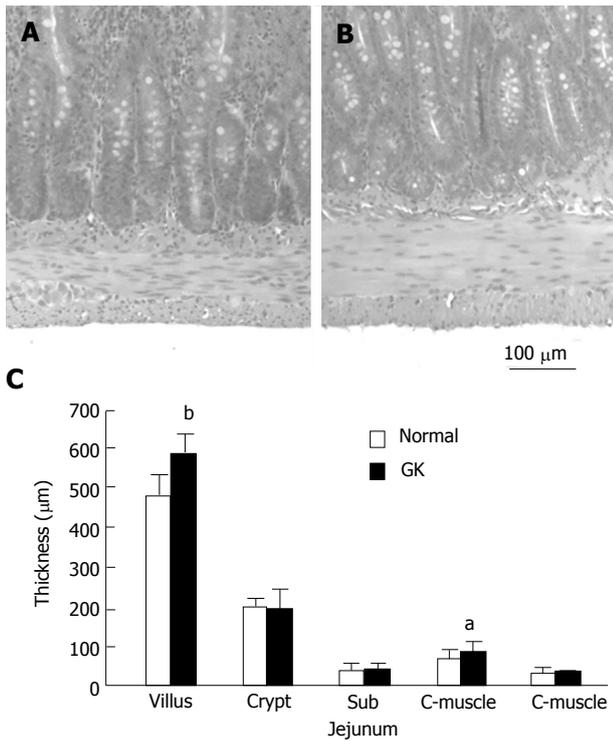
### General data

The body weight and blood glucose level were significantly higher in the GK group than those in the Normal group during the whole experimental period ( $P < 0.01$ , Table 1). The histological data are presented in Figure 3. Top figure represents examples of muscle layer thickness in the normal (Figure 3A) and GK (Figure 3B) rats. Compared with the normal rats, the muscle layer thickness was bigger in the GK rats. Bottom figure showed different layer thickness of jejunum between Normal and GK groups (Figure 3C). The villous height and circumferential muscle layer thickness of jejunal segments were higher in the GK group compared with the Normal group (villous height,  $571.3 \pm 34.8 \mu\text{m}$  vs  $475.1 \pm 44.1 \mu\text{m}$ ,  $P < 0.01$ ; circumferential muscle,  $68.9 \pm 11.9$

**Table 1** Body weight and blood glucose level

	Group	Rat age (wk)							
		18	20	22	24	26	28	30	32
Body weight (g)	GK	390.6 ± 20.2 <sup>b</sup>	408.1 ± 19.4 <sup>b</sup>	421.1 ± 22.1 <sup>b</sup>	431.7 ± 23.2 <sup>b</sup>	427.5 ± 24.3 <sup>b</sup>	436.1 ± 22.5 <sup>b</sup>	438.4 ± 21.6	443.5 ± 20.5 <sup>b</sup>
	Normal	350.3 ± 16.4	363.3 ± 17.3	371.3 ± 12.5	378.2 ± 15.1	386.8 ± 14.6	391.2 ± 14.3	397.3 ± 13.7	400.6 ± 17.2
Glucose level (mmol/L)	GK	8.89 ± 1.07 <sup>a</sup>	8.75 ± 0.32 <sup>a</sup>	9.15 ± 1.12 <sup>a</sup>	8.97 ± 0.42 <sup>a</sup>	8.86 ± 0.36 <sup>a</sup>	7.71 ± 0.44 <sup>a</sup>	7.92 ± 0.37 <sup>a</sup>	8.31 ± 0.38 <sup>a</sup>
	Normal	5.91 ± 0.43	6.06 ± 0.29	5.99 ± 0.57	5.93 ± 0.39	6.17 ± 0.54	5.57 ± 0.55	5.55 ± 0.36	5.75 ± 0.48

The body weight and blood glucose level of GK group were significantly higher than those of normal group during the whole experimental period. Compared with normal group, <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01. GK: Goto-Kakizaki.



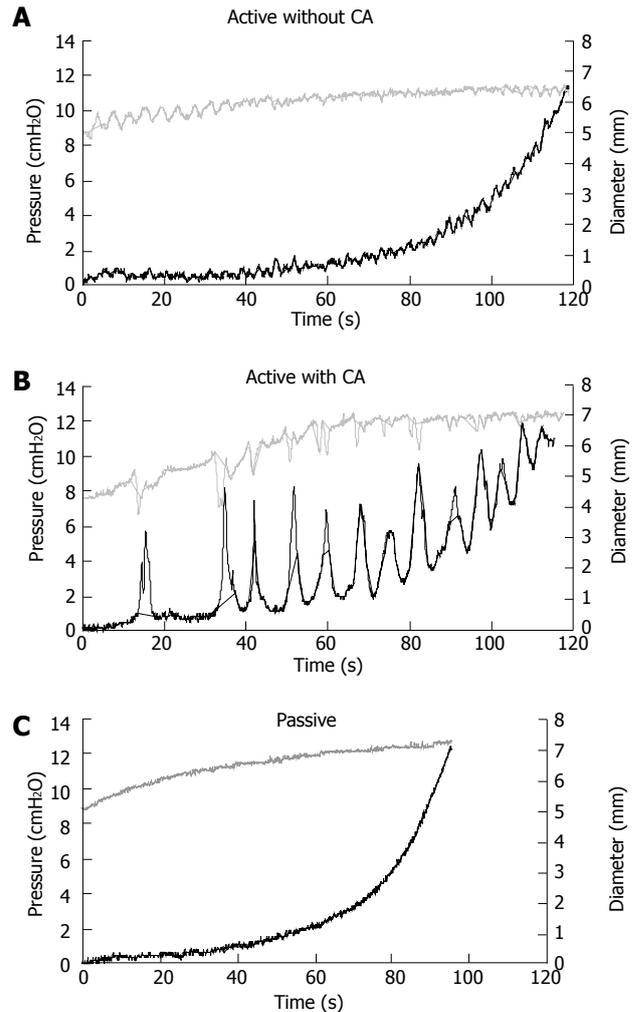
**Figure 3** Histological data. The top figure illustrates the example of jejunal muscle layer in Normal (A) and GK (B) rats. The increasing muscle layer thickness was noted in GK diabetic rat. The bottom figure (C) shows the jejunal layer thickness in two different groups. The villous height and circumferential muscle layer thickness were significantly bigger in GK group (Compared with normal group: <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01). GK: Goto-Kakizaki.

um *vs* 57.1 ± 6.4 um, *P* < 0.05). No significant difference was found for other layers (*P* > 0.05).

**Pressure-diameter curves**

Figure 4 illustrates ramp distension tracings of the pressure and diameter of jejunal segment in a normal rat with and without CA application. Waves of peristaltic contraction were clearly observed both from pressure and diameter curves (Figure 4A and B). The pressure increased whereas the diameter decreased during each contraction wave. Contractions were stronger after CA application (Figure 4B). Peristaltic contractions were not observed during distensions in calcium-free Krebs solution containing EGTA and papaverine (Figure 4C).

Figure 5 shows jejunal ramp distension pressure-diameter curves during CA application in normal (A) and GK diabetic (B) rats. The peak contraction amplitude is

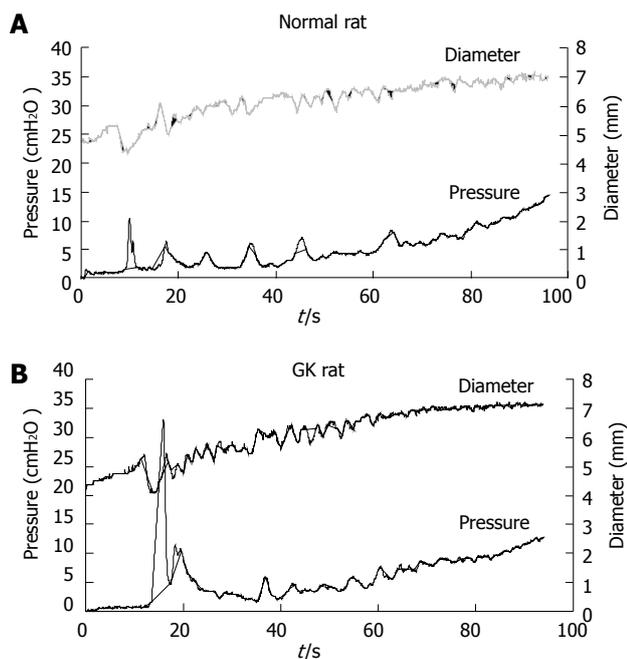


**Figure 4** Examples of pressure-diameter curves. Illustration of ramp distension curves of the pressure and diameter of jejunal segment with and without CA application obtained from a Normal rat. Waves of peristaltic contraction were clearly observed (top and middle). The smooth muscle contraction was abolished by papaverine (bottom). The CA application enhanced the peristaltic contraction (middle). CA: Carbachol.

higher in the GK diabetic rat than in the normal rat.

**Contraction thresholds**

The pressures (A), stresses (B) and strains (C) at the contraction threshold from the GK and Normal groups are shown in Figure 6. During CA application in the GK group, the pressure and stress thresholds were lower compared to the distensions before CA application (pressure threshold,



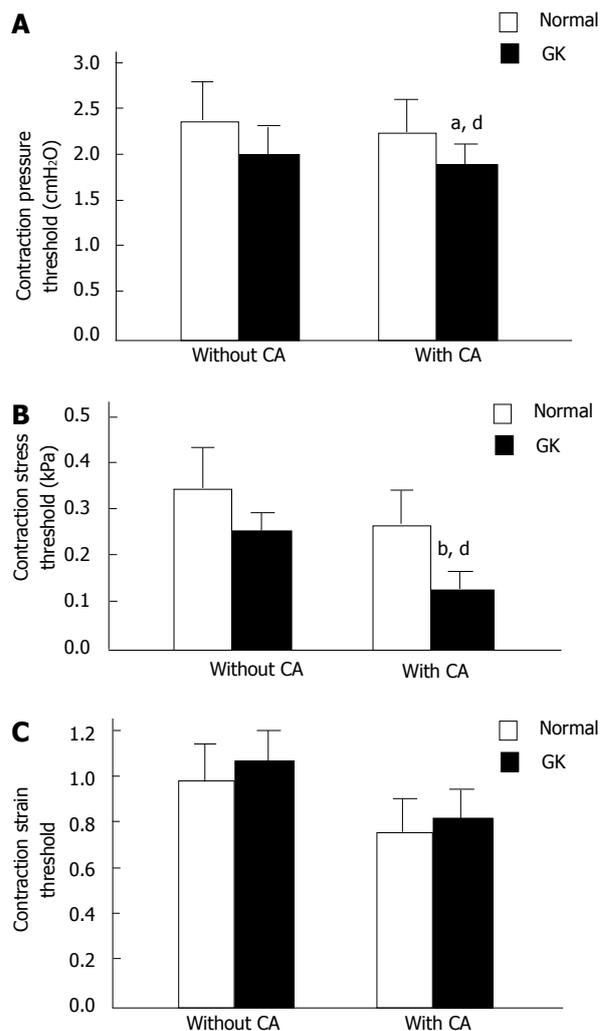
**Figure 5** Pressure-diameter curves from normal and goto-Kakizaki groups. Illustration of ramp distension curves of the pressure and diameter of jejunal segment with carbachol application obtained from a Normal (top) and a GK diabetic (bottom) rats. Higher peak of contraction was observed in the GK diabetic rat. CA: Carbachol; GK: Goto-Kakizaki.

$1.01 \pm 0.07$  vs  $1.99 \pm 0.19$  cm H<sub>2</sub>O,  $F = 14.870$ ,  $P = 0.003$ ; stress threshold,  $0.11 \pm 0.01$  vs  $0.24 \pm 0.02$  kPa,  $F = 16.474$ ,  $P = 0.002$ ). In the Normal group no difference was found before and during CA application ( $P > 0.05$ ). Compared with the Normal group, the pressure and stress thresholds were lowest in the GK group both for distensions before and during CA application. Significant difference was found during CA stimulation (GK group compared with Normal group, pressure threshold,  $1.01 \pm 0.07$  vs  $2.13 \pm 0.32$  cm H<sub>2</sub>O,  $F = 7.767$ ,  $P = 0.015$ ; and stress threshold,  $0.11 \pm 0.01$  vs  $0.25 \pm 0.03$  kPa,  $F = 13.624$ ,  $P = 0.003$ ). The strain at the contraction threshold did not differ between the GK and Normal groups ( $P > 0.05$ ), nor did strain at the contraction threshold differ before and during CA application in either of the two groups ( $P > 0.05$ ).

**Analysis of tonic and phasic stress-strain curves**

Total phasic (A), total tonic (B), active phasic (C) and active tonic (D) stresses as function of strains from GK and Normal groups before and during CA application are shown in Figure 7. The amplitude of total phasic, total tonic, active phasic and active tonic circumferential stresses did not differ before CA application. The total phasic and tonic stresses but not active phasic and tonic stresses increased during CA application in the GK group compared with those obtained in the Normal group (total phasic stress,  $4.56 \pm 0.68$  kPa vs  $3.74 \pm 0.47$  kPa,  $P < 0.05$ ; total tonic stress,  $3.42 \pm 0.56$  kPa vs  $2.92 \pm 0.34$  kPa,  $P < 0.05$ ). Furthermore, the maximum active phasic and active tonic stresses differed during CA application ( $2.51 \pm 0.45$  kPa vs  $1.42 \pm 0.27$  kPa,  $P < 0.01$ ).

The normalized active phasic (A) and tonic stresses

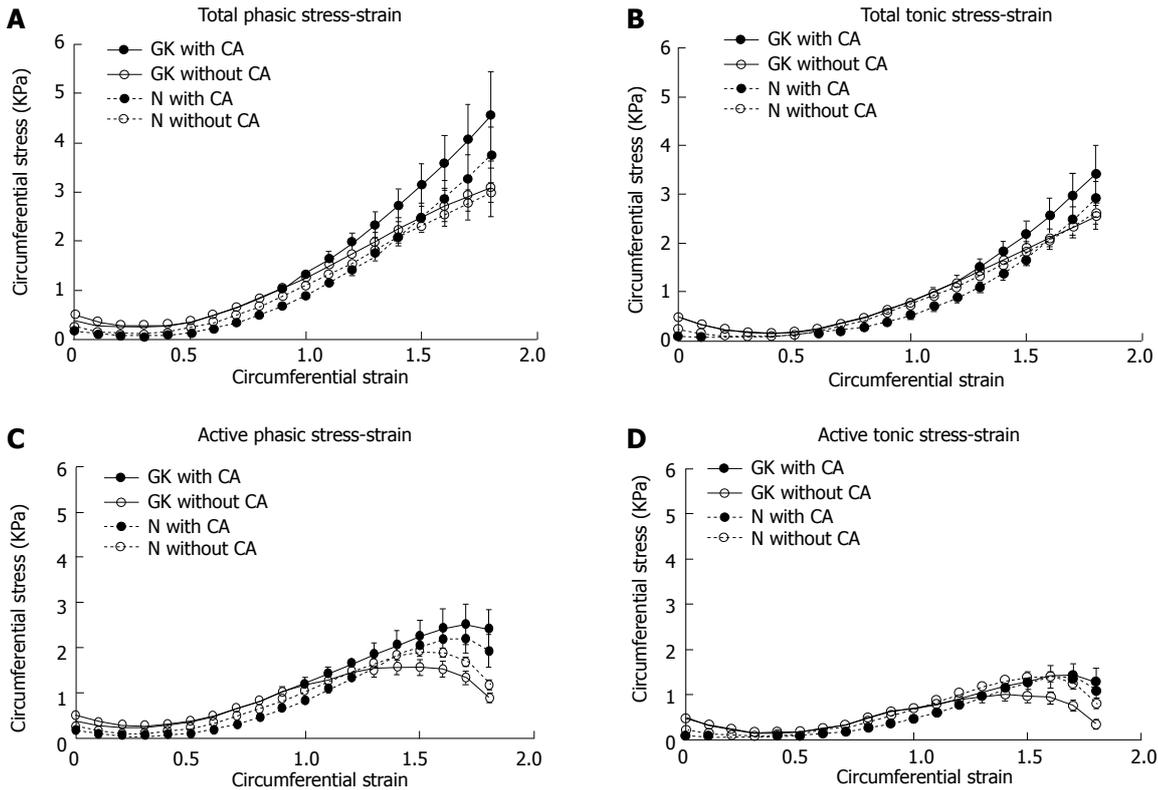


**Figure 6** Contraction thresholds. Illustration of the pressures (top), stresses (middle) and strains (bottom) at the contraction threshold in two different groups. The pressure and stress thresholds were significantly decreased in GK group after CA stimulation (compared with Normal group, pressure threshold, <sup>a</sup> $P < 0.05$ ; stress threshold, <sup>b</sup> $P < 0.01$ ). Furthermore, the pressure and stress thresholds were significantly decreased in GK group with CA application (compared with without CA, <sup>d</sup> $P < 0.01$ ) but not in Normal group ( $P > 0.05$ ). The strain at the contraction threshold did not differ between two different groups ( $P > 0.05$ ) and between before and after CA application ( $P > 0.05$ ). CA: Carbachol; GK: Goto-Kakizaki.

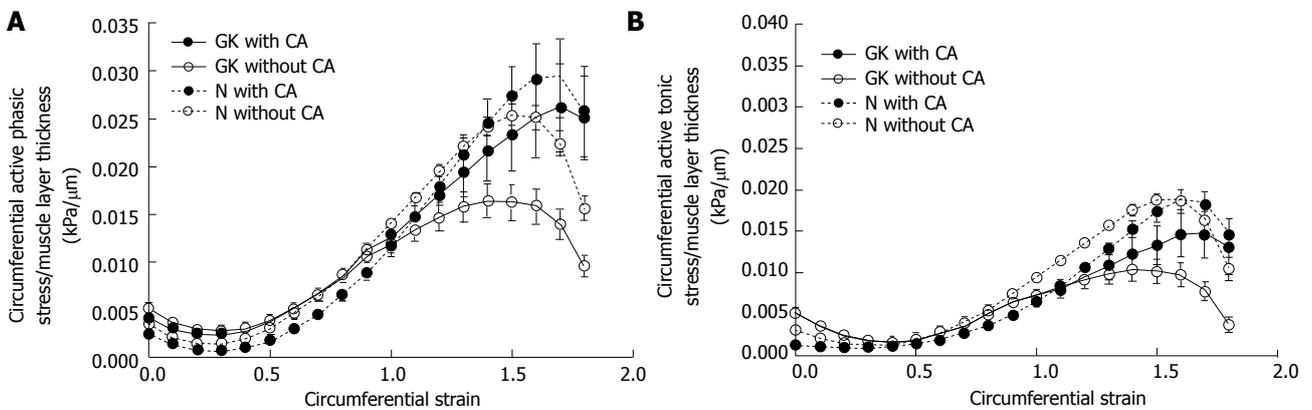
(B) as function of strains are shown in Figure 8. When normalized to muscle layer thickness, the amplitude of active phasic and tonic stresses were significantly lower before CA application in the GK group compared with the Normal group (normalized active phasic stress,  $0.0164 \pm 0.0018$  kPa/ $\mu$ m vs  $0.0254 \pm 0.0013$  kPa/ $\mu$ m,  $P < 0.05$ ; normalized active tonic stress,  $0.0103$  kPa/ $\mu$ m  $\pm 0.0014$  vs  $0.0187 \pm 0.0012$  kPa/ $\mu$ m,  $P < 0.05$ ) whereas they did not differ during CA application (normalized active phasic stress,  $0.0261 \pm 0.0047$  kPa/ $\mu$ m vs  $0.0292 \pm 0.0036$  kPa/ $\mu$ m,  $P > 0.05$ ; normalized active tonic stress,  $0.0148 \pm 0.0028$  kPa vs  $0.0186 \pm 0.0017$  kPa,  $P > 0.05$ ).

**DISCUSSION**

The length-tension diagrams known from physiological and pharmacological studies of smooth muscle strips *in*



**Figure 7 Phasic and tonic stresses as function of strains.** The total phasic (A) and tonic stresses (B) as function of strains are presented in the top and the computed active phasic (C) and tonic stresses (D) as function of strain in the bottom. The amplitude of total phasic, total tonic, active phasic and active tonic circumferential stresses did not differ without CA application but significantly increased after CA application in GK group compared those with Normal group ( $P < 0.05$ ). Furthermore, the maximum for the active phasic and active tonic stresses differed ( $P < 0.01$ ). CA: Carbachol; GK: Goto-Kakizaki.



**Figure 8 Normalized active phasic and tonic stresses as function of strains.** The normalized active phasic (top) and tonic stresses (bottom) illustrated as function of strains. When normalized to muscle layer thickness, the amplitude of active stresses significantly decreased without CA application ( $P < 0.05$ ) and did not differ after CA application ( $P > 0.05$ ). CA: Carbachol.

*in vitro*<sup>[25,33]</sup> can be reproduced in intact segment of intestine *in vitro* as shown in the present and previous studies<sup>[28,29]</sup>. The main findings of the current study were that the amplitudes of total phasic, total tonic, active phasic and active tonic circumferential stresses did not differ before CA application but the total phasic and total tonic stresses increased during CA application in GK diabetic rats. However, when normalized to muscle layer thickness, the active stress amplitudes decreased before CA application but did not differ during CA application in

the GK diabetic rats. Furthermore, the pressure and stress thresholds were significantly lower in the GK group compared with the Normal group during CA application.

The mechanical properties of the small intestine can be divided into passive properties arising from connective tissue element, tonic active properties reflecting baseline muscle activity, and phasic active property reflecting the contractile forces of distension-induced neuromuscular function. The passive and the active stress-

strain curves depend on the wall structure, the wall mechanical properties and the smooth muscle contractile properties. Recent studies demonstrated that histological and biomechanical remodeling of the small intestine, such as residual strain changes and intestinal wall stiffening, occurred in diabetic patients<sup>[17]</sup> and animals<sup>[19,20,23,34]</sup>. Such diabetes-induced remodeling may affect intestinal smooth muscle contractility.

Many human<sup>[1,35]</sup> and animal<sup>[19,20,35-37]</sup> studies have shown that diabetes causes structural changes of intestinal smooth muscle. Furthermore, several studies have demonstrated that function of intestinal smooth muscle also changed due to diabetes<sup>[36,38-42]</sup>. There are many ways to study smooth muscle contractility<sup>[43]</sup>. However from biomechanical point of view, muscle mechanical properties are best described in terms of stress and strain, *i.e.*, the force per area and tissue deformation<sup>[28,29]</sup>. Therefore, stress-strain data are important for understanding the mechanical function of remodeled smooth muscle in the diabetic intestine. Length-tension diagrams have been derived from the human gastric antrum and duodenum<sup>[32,44]</sup>, although butylscopolamine may not have abolished all phasic activity when analyzing the passive properties. The present and previous<sup>[28,29]</sup> *in vitro* studies produced tonic and phasic stress-strain curves referenced to the passive stress-strain curve because papaverine completely abolished all smooth muscle activity. Computation of the stress depends on the wall thickness which cannot easily be measured *in vivo*. However it can be measured *in vitro*.

In the present study the thicknesses of intestinal mucosa layer and muscle layer increased in diabetic rats. The increase in the muscle layer thickness is mainly due to circumferential muscle layer thickening and like caused by the increased circumferential stress. Before CA application, stress-strain data analysis of the contraction showed that the amplitude of total phasic, total tonic, active phasic and active tonic circumferential stresses as function of strains did not differ between diabetic and normal rats. However when normalized to muscle layer thickness, the amplitude of active phasic and tonic stresses significantly decreased. It indicates that the remodeled smooth muscle cells may be somehow altered due to long-term diabetes. The information regarding the direct effects of diabetes on ultrastructural changes of intestinal smooth muscle cell is very limited. However, one report indicated that streptozotocin-induced diabetes caused ultra-structural changes of venous and arterial smooth muscle cells which may account for the specific vascular complications<sup>[45]</sup>. Such changes may also occur in the diabetic intestinal smooth muscle and account for decreasing active contraction force per unit. This is needed to further study. However, the decreasing active contraction force per unit is likely to be compensated by increasing muscle layer thickness which is regulated in stress-dependent way. Furthermore, it is worthwhile to notice that the maximum of active phasic and active tonic stresses differ. This probably means that

they are regulated in different ways.

It is interesting at the present study to notice that after CA application, the amplitude of total phasic and total tonic stresses increased in the GK diabetic rats and the decreased amplitude of active stresses normalized to muscle layer thickness in the GK diabetic rats was corrected. Furthermore, the pressure and stress thresholds were significantly smaller in GK group compared with Normal group after CA application. All these together indicate that the intestinal muscle in the diabetic GK rats preserved their responses to the CA stimulation or even have higher sensitivity to CA application. It is well known that CA can directly stimulate muscarinic and nicotinic cholinergic receptors<sup>[30]</sup>. Therefore the receptor density likely has important impact on the GK diabetic intestinal smooth muscle cells. Although no direct evidence was obtained at present study, it was in one previous study demonstrated that the detrusor supersensitivity was observed after only 1 wk of untreated STZ-induced diabetes in the rat. The overactivity was associated with an enhanced sensitivity to carbachol, which could be partly explained by an increase in receptor density<sup>[46]</sup>. It maybe the same case in the GK diabetic intestine and need to identify in the future. But the controversial results were also reported on diabetic colon<sup>[47]</sup> and gastric antrum<sup>[48]</sup>. The former study has been shown that the contractility of the proximal colon in response to CA was weaker in the diabetic rats<sup>[47]</sup>. The later study demonstrated that gastric antral smooth muscle cells from streptozotocin-induced rats and db/db spontaneously diabetic mice impair their contractile response to CA<sup>[48]</sup>. These controversial results may indicate the different regions of gastrointestinal tract in the diabetes have different reaction to the CA stimulation.

In summary, the force (stress) generated by the smooth muscle per unit was decreased in GK diabetic rats and it is likely to be compensated by smooth muscle proliferation. It is indicating that the intestine remodels in a stress-dependent way. Furthermore, the smooth muscle in GK diabetic intestine preserves its response to the stimulation of CA same as the smooth muscle in the normal small intestine.

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## COMMENTS

### Background

The intestinal motility disorders have been found both in the diabetic patients and animals. However, the pathogenesis of intestinal motility disorders in diabetes mellitus is complex and is not well understood. The morphological and biomechanical remodeling of gastrointestinal tract occurred in the diabetes which may change the smooth muscle function, and then affect the intestinal motility.

### Research frontiers

There are many ways to study smooth muscle contractility, however from biomechanical point of view, muscle mechanical properties are better to be

described in terms of stress and strain, *i.e.*, the force per area and tissue deformation. Therefore, stress-strain data are important for understanding the mechanical function of remodeled smooth muscle in the diabetic intestine.

### Innovations and breakthroughs

The present *in vitro* study produced tonic and phasic stress-strain curves referenced to the passive stress-strain curve because papaverine completely abolished all smooth muscle activity. Computation of the stress depends on the wall thickness which cannot be directly measured *in vivo*. However it can be measured *in vitro*. Furthermore, the authors use carbachol as stimulator which is a parasympathomimetic drug that directly stimulates cholinergic receptors.

### Applications

The mechanical properties of the small intestine can be divided into passive property, tonic active property and phasic active property. By understanding how the phasic and tonic properties of intestinal smooth muscle are changed in the diabetes, this study may represent a future strategy for studying the intestinal smooth function in different diseases.

### Terminology

Stress is the force per area and strain is the tissue deformation. Stress-strain data are important for understanding the mechanical function of smooth muscle. Carbachol is a parasympathomimetic drug that directly stimulates muscarinic and nicotinic cholinergic receptors. The receptor density has important impact on the function of smooth muscle cells.

### Peer review

The main goal of this paper was to examine whether the function of jejunal smooth muscle was altered during diabetes. To do this, the authors proposed to generate phasic and tonic stress-strains curves in normal Wistar and diabetic rats before and after parasympathetic stimulation with carbachol. The experimental plan seems to be sound and follows a logical progression.

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## Prevalence of normoglycemic, prediabetic and diabetic A1c levels

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### Abstract

**AIM:** To investigate normoglycemic, prediabetic and diabetic A1c levels in those with prediabetes; and prediabetic and diabetic A1c levels in those with non-prediabetes.

**METHODS:** The National Health and Nutritional Examination Survey (NHANES) 2007-2008 and NHANES 2009-2010 were utilized to examine and compare trends and differences among five different ethnic groups (Mexican Americans, Other Hispanics, Non-Hispanic Whites, Non-Hispanic Blacks, Other/Multi-racials) with normoglycemic, prediabetic and diabetic A1c levels with self-reported prediabetes and prediabetic and diabetic A1c levels in those with self-reported non-prediabetes. Sample participants of the five ethnic groups were limited to those 20 years of age and older, who had completed the diabetes questionnaire and had A1c measured. Descriptive statistics were computed for all variables.  $\chi^2$  were performed on all five ethnic groups to examine significant differences of normoglycemic, prediabetic and diabetic A1c levels in those with self-reported prediabetes, and prediabetic and diabetic A1c levels in those with self-reported non-prediabetes.

**RESULTS:** This study demonstrates that of the five different ethnic groups from NHANES 2007-2008 to NHANES 2009-2010, Non-Hispanic Whites (6.5% increase) and Non-Hispanic Blacks (0.2% increase) were the only two groups with an increase in the number of self-reported prediabetes. Although the overall percentage of Mexican Americans who self-reported prediabetes had remained the same (5%) from NHANES 2007-2008 to NHANES 2009-2010,  $\chi^2$  analysis showed significant differences when examining the different ranges of A1c levels (normoglycemic, prediabetic and diabetic). Among Mexican Americans who self-reported prediabetes, normoglycemic ( $P = 0.0001$ ) and diabetic ( $P = 0.0001$ ) A1c levels from NHANES 2007-2008 to NHANES 2009-2010. For Non-Hispanic Whites who self-reported prediabetes, prediabetic ( $P = 0.0222$ ); and diabetic ( $P \leq 0.0001$ ) A1c levels from NHANES 2007-2008 to NHANES 2009-2010. For Non-Hispanic Blacks who self-reported prediabetes, there were significant differences ( $P = 0.0001$ ) for all A1c levels (normoglycemic, prediabetic and diabetic A1c levels). For Other/Multi-racials with self-reported prediabetes there was significant differences in those with normoglycemic ( $P = 0.0104$ ) and diabetic ( $P = 0.0067$ ) A1c levels from NHANES 2007-2008 to NHANES 2009-2010. For all combined ethnic groups who self-reported not having prediabetes (non-prediabetes), 19.9% of those in NHANES 2007-2008 and 22.4% in the NHANES 2009-2010 showed to have prediabetic A1c levels. When separately examining each of the five ethnic groups who self-reported not having prediabetes, all showed an increase in those with prediabetic A1c levels from NHANES 2007-2008 to NHANES 2009-2010. Through  $\chi^2$  analysis, all five ethnic groups who self-reported not having prediabetes showed significant differences ( $P < 0.0001$ ) in all A1c levels (normoglycemic, prediabetic and diabetic) from NHANES 2007-2008 to NHANES 2009-2010.

**CONCLUSION:** The findings highlight the need of prediabetes awareness and of education in the community

as ways to reduce the number of people with prediabetes.

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**Key words:** National Health and Nutritional Examination Survey; Prediabetes; Undiagnosed prediabetes; Undiagnosed diabetes

**Core tip:** The National Health and Nutritional Examination Survey (NHANES) 2007-2008 and NHANES 2009-2010 data sets were used. In these data sets, trends and differences in normoglycemic, prediabetic and diabetic A1c levels among five different ethnic groups are explained in detail. Significance of these findings, limitations of these data sets, and community-based initiatives are discussed.

Aponte J. Prevalence of normoglycemic, prediabetic and diabetic A1c levels. *World J Diabetes* 2013; 4(6): 349-357 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/349.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.349>

## INTRODUCTION

Prediabetes affects an estimated 79 million adults in the United States<sup>[1]</sup>. It is projected that by 2020, 52% of United States adults will have either prediabetes or diabetes<sup>[2]</sup>. Prediabetes is usually asymptomatic and is defined as “blood glucose levels higher than normal but not high enough for diabetes”<sup>[3]</sup>. A report from the Centers for Disease Control and Prevention (CDC)<sup>[4]</sup> indicated that only 11% of those with prediabetes are aware of having it. According to the American Diabetes Association<sup>[5]</sup>, 65% of those with diabetes were aware of having it.

There are three methods [*i.e.*, fasting plasma glucose (FPG), glycosylated hemoglobin (A1c) and 2-h oral glucose tolerance test] used to measure prediabetes and diabetic ranges, but the most common clinical tool used to measure and test blood glucose levels for diagnosing diabetes (*i.e.*, A1c  $\geq 6.5\%$ ) in non-pregnant adults is (A1c)<sup>[6]</sup>. According to the National Institute of Diabetes and Digestive and Kidney Diseases<sup>[7]</sup>, A1c is “a blood test that provides information about a person’s average levels of blood glucose over the past 3 mo.” Prediabetes is also known as impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT), which are the leading risk factors for diabetes<sup>[8]</sup>. Of those with prediabetes, 15%-30% will progress and develop diabetes within 5 years<sup>[9]</sup>. But, if a person with prediabetes makes lifestyle changes (*i.e.*, lose weight and increase physical activity), blood glucose levels could potentially return to normal. For example, the Diabetes Prevention Program (DPP) showed that people with prediabetes can often prevent or delay diabetes if a person loses a modest amount of weight (*i.e.*, 5%-7% of body weight) and increases physical activity, allowing blood glucose levels to return to

normal<sup>[10]</sup>. The DPP study and other studies have shown that with lifestyle changes, prediabetes could be reversed and diabetes avoided. Hence, early diagnosis, treatment and management of prediabetes could avoid, reduce or delay its progression to diabetes and diabetes-related complications (*e.g.*, heart disease and stroke).

The number of people with diabetes in the United States is rapidly growing, the ethnic diversity is changing, and there is a disproportionate prevalence of diabetes in some ethnic groups. It is important to know if this is the same for prediabetes. Data on prediabetes is scarce, which makes knowing the prevalence of those with prediabetes necessary, including those with reversed prediabetes, and undiagnosed diabetes, including differences among ethnic/racial groups. Another important trend to examine are those with undiagnosed prediabetes. Table 1 has the definitions of diagnosed, undiagnosed and reversed prediabetes, and undiagnosed diabetes.

As prediabetes is detectable, treatable and reversible, it is especially important that its trends be monitored in diverse populations. Also, given that prediabetes is a risk factor for diabetes, it is vital to examine trends of those with undiagnosed diabetes among different ethnic/racial groups. Therefore, descriptive data on United States Non-Hispanic Whites, Non-Hispanic Blacks, Mexican Americans, Other Hispanics, and Other/Multi-racial groups with prediabetes presents vital information regarding trends and differences in the prevalence of reversed, diagnosed and undiagnosed prediabetes, and undiagnosed diabetes, including the distribution by ethnicity among these groups.

## Literature review

Three different PUBMED searches were conducted of articles published from January 2003 to June 2013. One search used the key words prediabetes, which identified 2094 studies. Another used the keywords prediabetes and ethnic groups, which identified 73 articles. Of those 73 results, 59 were studies conducted in the United States, and 14 were conducted outside the United States. Of the 59 United States studies, 28 included more than one ethnic group, and of the 14 non-United States studies, 8 included more than one ethnic group. The keywords prediabetes and ethnic groups and race identified 45 studies, and the keywords prediabetes and ethnic groups and race and undiagnosed identified 4 studies, all of which were part of the 73 identified from the previous keyword search. Hence, these 4 studies were the final articles considered for this review.

One study using NHANES data reported findings of metabolic syndrome on four ethnic groups (*i.e.*, Non-Hispanic White, Non-Hispanic Black, Hispanics and Others)<sup>[11]</sup>. A second study using NHANES reported prediabetes trends from 1988-2008 among different ethnic groups (*i.e.*, Non-Hispanic Black, Non-Hispanic White, and Mexican American/Other Hispanic)<sup>[12]</sup>. The study reported that Non-Hispanic Blacks had a significantly higher prediabetes rate than Non-Hispanic

**Table 1** Definitions of current study variables within different groups

Different groups	Responses and A1c levels
Prediabetes group	
Self-reported Prediabetes	“Yes” response to “ever told you have prediabetes,” and a “no” response to “doctor told you have diabetes.”
Reversed prediabetes	Self-reported “yes” response to “ever told you have prediabetes,” “no” response to “doctor told you have diabetes” and have normoglycemic A1c levels (< 5.7%).
Diagnosed prediabetes	Self-reported “yes” response to “ever told you have prediabetes,” “no” response to “doctor told you have diabetes” and have prediabetic A1c levels (5.7%-6.4%).
Undiagnosed diabetes	Self-reported “yes” response to “ever told you have prediabetes,” “no” response to “doctor told you have diabetes” and have diabetic A1c levels ( $\geq 6.5\%$ ).
Non-Prediabetes group	
Self-reported non-prediabetes	“No” response to “ever told you have prediabetes,” and a “no” response to “doctor told you have diabetes.”
Not having prediabetes	Self-reported “no” response to “ever told you have prediabetes,” “no” response to “doctor told you have diabetes” and have normoglycemic A1c levels (< 5.7%).
Undiagnosed prediabetes	Self-reported “no” response to “ever told you have prediabetes,” “no” response to “doctor told you have diabetes” and have prediabetic A1c levels (5.7%-6.4%).
Undiagnosed diabetes	Self-reported “no” response to “ever told you have prediabetes,” “no” response to “doctor told you have diabetes” and have diabetic A1c levels ( $\geq 6.5\%$ ).

Whites, but Mexican Americans and Other Hispanic rates were not significantly higher than Non-Hispanic Whites. Unlike the study by Sentell *et al*<sup>[12]</sup> the current study includes the most recent NHANES data currently available, examining NHANES 2007-2008 and NHANES 2009-2010 data of those with prediabetes, undiagnosed prediabetes and undiagnosed diabetes among five specific ethnic/racial groups (Non-Hispanic Whites, Non-Hispanic Blacks, Mexican Americans, Other Hispanics, and Other/Multiracial).

### Purpose

The purpose of this article is twofold: (1) to investigate A1c levels for normoglycemia, prediabetes and diabetes prevalence in those with self-reported prediabetes; and (2) to investigate A1c levels for prediabetes and diabetes in those with self-reported non-prediabetes (*i.e.*, self-report indicates has not ever had prediabetes).

This study will begin to address a research gap by reporting on the results of a secondary analysis of A1c levels among five ethnic groups (Non-Hispanic Whites, Non-Hispanic Blacks, Mexican Americans, Other Hispanics and Other/Multiracials) who self-reported prediabetes and who self-reported non-prediabetes, in order to discuss methods of enhancing community outreach in this population. While further research on prediabetes is essential, the findings in this article also discusses community initiatives that health care providers can implement to integrate prediabetes awareness, care and management goals into practice for their patients with prediabetes.

### Research hypotheses

This study's hypotheses were as follows: (1) There are significant differences between normoglycemic, prediabetic and diabetic A1c levels among five different ethnic groups (Non-Hispanic Whites, Non-Hispanic Blacks, Mexican Americans, Other Hispanics and Other/Multiracial) with self-reported prediabetes; and (2) There are significant differences between prediabetic and diabetic

A1c levels among the five different ethnic groups (Non-Hispanic Whites, Non-Hispanic Blacks, Mexican Americans, Other Hispanics and Other/Multiracial) with self-reported non-prediabetes.

## MATERIALS AND METHODS

The National Center for Health Statistics (NCHS), which is part of the CDC, designs, develops and maintains health-related systems and surveys; and collects United States demographic and health and nutritional data<sup>[13]</sup>. Among its surveys are NHANES 2007-2008 and NHANES 2009-2010. As part of each survey design, NCHS provides weights for researchers to use for data analysis; the method of usage for NHANES 2007-2008 is in the Analytic and Reporting Guidelines. NHANES design, survey methods, examination and laboratory techniques, and sample selection have been reported previously in detail<sup>[14]</sup>.

### Sample

For NHANES 2007-2008 and NHANES 2009-2010, data were collected on a probability sample of the entire civilian, non-institutionalized United States population categorized by ethnic group: Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black and Other/Multiracial.

Both NHANES surveys contained questionnaire and laboratory data. Secondary analysis of the raw data utilized the sample weight representing the sample population provided by NHANES 2007-2008 and NHANES 2009-2010 surveys. Details on the NHANES sample procedures are outlined in Aponte<sup>[14]</sup>.

### Sample selection procedures

For the current study, participants were limited to members of each of the five ethnic groups, 20 years of age and older, who had completed the diabetes questionnaire and had A1c measured. The demographic variables of age and gender were also included in this study but not

identified as risk factors. The current study utilized a number of different self-reported data. Table 1 provides the definitions of variables for two groups: those with self-reported prediabetes and non-prediabetes. Both questions were in the diabetes questionnaires of the NHANES surveys. The prediabetes question “ever told you have prediabetes” included the terms IFG, IGT or borderline diabetes in the question and these terms were explained as “blood sugar (glucose) level that are higher than normal but not high enough to be diabetes”. In addition, the prediabetes question is not specific to the current prediabetes status. In order to ensure that these participants had not converted to having diabetes, a diabetes question (*i.e.*, “doctor told you have diabetes”) was included in the data analysis. Only those who responded “no” to this diabetes question were included in all of the analysis of this current study.

Additional self-reported data were: age, ethnicity, pregnancy status from questionnaires, as well as A1c levels collected from laboratory findings. According to the ADA<sup>[6]</sup>, an A1c of less than 5.7% means normoglycemic levels (*i.e.*, no prediabetes or diabetes); 5.7% to 6.4% is prediabetes, and 6.5% and greater means diabetes. Normoglycemic, prediabetic and diabetic A1c levels were examined in both the self-reported prediabetes and non-prediabetes groups.

A1c is used to diagnose prediabetes and diabetes in non-pregnant adults. To ensure the sample were not pregnant, a reproductive question regarding pregnancy status was included in data analysis. In both the NHANES 2007-2008 and NHANES 2009-2010 surveys, the reproductive question (*i.e.*, “are you pregnant now?”) was only asked of women 20 to 59 years of age. This reproductive question was not asked of women 60 years and older or of men, and for this reason the question was only included in the data analysis for women 20-59 years of age. To ensure all participants in the current study were non-pregnant adults, each of the following ways to stratify were used in the analysis for both groups (prediabetes and non-prediabetes): (1) self-reported prediabetes or self-reported non-prediabetes, and women 20 to 59 years of age who responded “no” to the reproductive health question; (2) self-reported prediabetes or self-reported non-prediabetes and all adult men; or (3) self-reported prediabetes or self-reported non-prediabetes in women 60 years of age and older. This created a total of three groups in those who self-reported prediabetes and three groups in those who self-reported non-prediabetes. The three groups in each category (self-reported prediabetes and non-prediabetes) were combined for the analysis of this current study.

Secondary analysis of NHANES 2007-2008 and NHANES 2009-2010 raw data was approved with exempt status by the Hunter College Human Research Protection Program Office, Institutional Review Board of Hunter College, City University of New York.

### Data collection

Original collection measures: Data collection for NHANES 2007-2008 and NHANES 2009-2010 occurred through personal interviews (initial questionnaire component);

and health examinations (supplemental questionnaires, physical examination and laboratory component).

The NHANES 2007 to 2008 and NHANES 2009 to 2010 phase 1 consisted of two questionnaires: (1) sample questionnaire, which included questions on individual health status, including self-report of prediabetes and diabetes diagnosis; and (2) family questionnaire, which included self-reported questions on family members.

The health examinations were conducted in a mobile examination center, where each participant was physically examined. A laboratory component included blood tests of A1c levels for diabetes risk and status. Other questionnaires included information on physical activity, tobacco use, prostate conditions for men, reproductive history for women, sexual activity, alcohol consumption, and drug use.

The method used to administer the questions in phase 2 was the same as the method used to collect NHANES 2007 to 2010 data, as previously described by Aponte<sup>[14]</sup>. The NHANES 2007 to 2008 and NHANES 2009 to 2010 data are stored by NCHS and can be downloaded for review and analysis.

Current study collection measures: For the current study, raw data from the NHANES 2007 to 2008 and NHANES 2009 to 2010 data sets were used to examine ethnicity (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black, Other/Multi racial) and A1c levels.

For descriptive analysis, A1c levels were examined in two groups independently, those who: (1) self-reported prediabetes; and (2) non-prediabetes. To facilitate analysis, numerical values differentiating normoglycemic (< 5.7%) from prediabetic (5.7%-6.4%) or diabetic levels ( $\geq$  6.5%) were created.

### Statistical analysis

All data analysis was conducted by utilizing NCHS’s recommended weights, available in the NHANES 2007-2008 and NHANES 2009-2010<sup>[15]</sup> data sets. The NHANES 2007-2008 and NHANES 2009-2010 data on diabetes, reproductive interview and laboratory data were reported as overall raw data without statistical analysis. Descriptive statistics were computed for all variables to examine the prevalence of normoglycemic, prediabetic and diabetic ( $\geq$  6.5%) A1c levels among five ethnic groups with self-reported prediabetes, and prediabetic and diabetic A1c levels in those with self-reported non-diabetes.  $\chi^2$  of independence were used to compute significance differences among all five ethnic groups. The significance level used in this current study was  $P < 0.05$ . Findings reported are those that were or were not significant. In this secondary analysis, the raw data were analyzed by using the statistical software product SAS version 9.3<sup>®</sup> (SAS Institute Inc, Cary, NC).

## RESULTS

### Self-reported prediabetes

Descriptive analysis: The total weighted sample of NH-

**Table 2 United States Population 20 years and older with self-reported prediabetes *n* (%)**

Glycated hemoglobin (A1c)	Self-report of: "Yes" ever being told they have prediabetes; and "No" being told they had diabetes	
	NHANES 2007-2008 Weighted	NHANES 2009-2010 Weighted
Normoglycemic levels (< 5.7%) reversed prediabetes	3565229/6566166 (54.3)	3281804/7569333 (43.4)
Prediabetic levels (5.7%-6.4%) prediabetes	2435163/6566166 (37.1)	3071030/7569333 (40.6)
Diabetic levels (> 6.5%) undiagnosed/unaware diabetes	564774/6566166 (8.6)	1216499/7569333 (16.0)
Total	<i>n</i> = 6566166	<i>n</i> = 7569333

**Table 3 Percentage of different ethnic groups 20 years and older with self-reported prediabetes *n* (%)**

Ethnicity	NHANES 2007-2008			NHANES 2009-2010		
	Weighted	SD		Weighted	SD	<i>P</i>
Mexican American	325142/6566166 (5.0)			379370/7569333 (5.0)		
Normoglycemic A1c (< 5.7%)	102664/325142 (31.6)	0.33		154730/379370 (40.8)	0.43	< 0.0001
Prediabetic A1c (5.7%-6.4%)	140099/325142 (43.1)	0.46		161539/379370 (42.6)	0.43	< 0.0001
Diabetic A1c (≥ 6.5%)	82379/325142 (25.3)	0.44		63101/379370 (16.6)	0.22	0.8521
Other Hispanic	172116/6566166 (2.6)			175097/7569333 (2.3)		
Normoglycemic A1c (< 5.7%)	94137/172116 (54.7)	0.16		104638/175097 (59.8)	0.43	0.3841
Prediabetic A1c (5.7%-6.4%)	73430/172116 (42.7)	0.43		56802/175097 (32.4)	0.42	0.3472
Diabetic A1c (≥ 6.5%)	4549/172116 (2.6)	0.36		13657/175097 (7.8)	0.12	0.9457
Non-Hispanic White	4757294/6566166 (72.5)			5976593/7569333 (79.0)		
Normoglycemic A1c (< 5.7%)	2571794/4757294 (54)	0.46		2696814/5976593 (45.1)	0.4	0.7869
Prediabetic A1c (5.7%-6.4%)	1872875/4757294 (39.4)	0.43		2311252/5976593 (38.7)	0.4	0.0222
Diabetic A1c (≥ 6.5%)	312625/4757294 (6.6)	0.23		968527/5976593 (16.2)	0.09	0.0024
Non-Hispanic Black	645476/6566166 (9.8)			757648/7569333 (10)		
Normoglycemic A1c (< 5.7%)	269742/645476 (41.8%)	0.46		212317/757648 (28%)	0.44	< 0.0001
Prediabetic A1c (5.7%-6.4%)	210513/645476 (32.6%)	0.42		483049/757648 (63.8%)	0.44	< 0.0001
Diabetic A1c (≥ 6.5%)	165221/645476 (25.6%)	0.43		62282/757648 (8.2%)	0.16	< 0.0001
Other/Multi-Racial	666138/6566166 (10.1%)			280625/7569333 (3.7%)		
Normoglycemic A1c (< 5.7%)	527892/666138 (79.2%)	0.27		113305/280625 (40.3%)	0.41	0.0104
Prediabetic A1c (5.7%-6.4%)	138246/666138 (20.8%)	0.27		58388/280625 (20.8%)	0.41	0.7737
Diabetic A1c (≥ 6.5%)	0/666138 (0.0%)	0.00		108932/280625 (38.8%)	0.13	0.0067
Total	<i>n</i> = 6566166			<i>n</i> = 7569333		

SD: Shine-dalgarnosequence.

ANES 2007-2008 (*n* = 6566166) and NHANES 2009-2010 (*n* = 7569333) comprised all participants aged 20 years and older with self-reported prediabetes (Table 2). For those with self-reported prediabetes, normoglycemic (< 5.7%), prediabetic (5.7%-6.4%) and diabetic (≥ 6.5%) A1c levels were examined. Of the total weighted sample in NHANES 2007-2008 and NHANES 2009-2010, there was a rise in the percentage of those participants with prediabetes (37.1% and 40.6%, respectively) and diabetic (8.6% and 16%, respectively) A1c levels, and a decrease in those with normoglycemic A1c levels (54.3% and 43.4%, respectively) (Table 2). When examining ethnic groups in NHANES 2007-2008 and NHANES 2009-2010, Non-Hispanic Whites (72.5% and 79%, respectively) had the largest growing percentage of self-reported prediabetes, followed by Non-Hispanic Blacks (9.8% and 10%, respectively). Other/Multi-racials (19.1% and 3.7%, respectively) and Other Hispanics (2.6% and 2.3%, respectively) were two ethnic groups who had a reduction in the number of people with self-reported prediabetes from NHANES 2007-2008 to NHANES 2009-2010. Mexican Americans were the only ethnic

group in which the combined percentage of normoglycemic, prediabetic and diabetic A1c levels, remained at 5% in both NHANES 2007-2008 and NHANES 2009-2010 surveys (Table 3).

$\chi^2$ : When analyzing each A1c level separately, there were differences in normoglycemic (< 5.7%), prediabetic (5.7%-6.4%) and diabetic (≥ 6.5%) A1c levels among the five ethnic groups with self-reported prediabetes in NHANES 2007-2008 and NHANES 2009-2010. There was a significant increase in the proportion of those Mexican Americans with self-reported prediabetes and a normoglycemic (< 5.7%) A1c level (31.6% and 40.8%, respectively, *P* ≤ 0.0001); and a significant decrease for those with prediabetic (5.7%-6.4%) A1c levels (43.1% and 42.6%, respectively, *P* ≤ 0.0001) from the NHANES 2007-2008 to NHANES 2009-2010 surveys. Although not significant, there was a slight increase in the proportion of those Other Hispanics with self-reported prediabetes who had a normoglycemic (< 5.7%) (54.7% and 59.8%, respectively, *P* = 0.3841) and diabetic (≥ 6.5%) (2.6% and 7.8%, respectively, *P* = 0.9457) A1c levels. For

**Table 4 United States population 20 years and older with self-reported non-prediabetes *n* (%)**

Glycated hemoglobin (A1c)	Self-report of: "No" ever being told they have prediabetes; and "No" being told they had diabetes	
	2007-2008 weighted	2009-2010 weighted
Normoglycemic levels (< 5.7%) no prediabetes or diabetes	128918166/164914962 (78.2)	122536437/161120813 (76.0)
Prediabetic levels (5.7%-6.4%) undiagnosed/unaware prediabetes	32926568/164914962 (19.9)	36065705/161120813 (22.4)
Diabetic levels (≥ 6.5%) undiagnosed/unaware diabetes	3070228/164914962 (1.9)	2518671/161120813 (1.6)
Total	<i>n</i> = 164914962	<i>n</i> = 161120813

**Table 5 Different ethnic groups 20 years and older with self-reported non-prediabetes *n* (%)**

NHANES 2007-2008 Ethnicity	Weighted	NHANES 2009-2010	
		Weighted	<i>P</i>
Mexican American	15104701/164914962 (9.2)	15411565/161120804 (9.6)	
Normal (A1c ≤ 5.7%)	12524514/15104701 (82.9)	11245974/15411161 (73)	< 0.0001
Prediabetes (A1c = 5.7%-6.4%)	2248885/15104701 (14.9)	3608202/15411161 (23.4)	< 0.0001
Diabetes (A1c ≥ 6.5%)	331302/15104701 (2.2)	557389/15411161 (3.6)	< 0.0001
Other Hispanic	8878061/164914962 (5.4)	8645012/161120804 (5.4)	
Normal (A1c ≤ 5.7%)	7011142/8878061 (78.9)	6519914/8645012 (75.4)	< 0.0001
Prediabetes (A1c = 5.7%-6.4%)	1655906/8878061 (18.7)	1950442/8645012 (22.6)	< 0.0001
Diabetes (A1c ≥ 6.5%)	211013/8878061 (2.4)	174656/8645012 (2)	< 0.0001
Non-Hispanic White	113923737/164914962 (69.1)	109714993/161120804 (68.1)	
Normal (A1c ≤ 5.7%)	90029378/113923737 (79)	85687554/109714993 (78.1)	< 0.0001
Prediabetes (A1c = 5.7%-6.4%)	22273553/113923737 (19.6)	22770821/109714993 (20.8)	< 0.0001
Diabetes (A1c ≥ 6.5%)	1620806/113923737 (1.4)	1256618/109714993 (1.1)	< 0.0001
Non-Hispanic Black	17698774/164914962 (10.7)	17766570/161120804 (11)	
Normal (A1c ≤ 5.7%)	12238510/17698774 (69.1)	11922107/17766570 (67.1)	< 0.0001
Prediabetes (A1c = 5.7%-6.4%)	4864294/17698774 (27.5)	5436479/17766570 (30.6)	< 0.0001
Diabetes (A1c ≥ 6.5%)	595970/17698774 (3.4)	407984/17766570 (2.3)	< 0.0001
Other/Multi-Racial	9309689/164914962 (5.6)	9582664/161120804 (5.9)	
Normal (A1c ≤ 5.7%)	7114622/9309689 (76.4)	7160888/9582664 (74.7)	< 0.0001
Prediabetes (A1c = 5.7%-6.4%)	1883930/9309689 (20.2)	2299761/9582664 (24)	< 0.0001
Diabetes (A1c ≥ 6.5%)	311137/9309689 (3.4)	112015/9582664 (1.2)	< 0.0001
Total	<i>n</i> = 164914962	<i>n</i> = 161120804	

Non-Hispanic Whites with prediabetes, there was a significant decrease in those with a prediabetic (5.7%-6.4%) (39.4% and 38.7%, respectively, *P* = 0.0222) A1c levels and a significant decrease in those with diabetic (≥ 6.5%) (6.6% and 16.2%, respectively, *P* = 0.0024) A1c levels. There was a significant increase in prediabetic (5.7%-6.4%) (32.6% and 63.8%, respectively, *P* ≤ 0.0001) A1c levels for Non-Hispanic Blacks with self-reported prediabetes, and significant decreases in those with normoglycemic (< 5.7%) (41.8% and 28%, respectively, *P* ≤ 0.0001) and diabetic (≥ 6.5%) (25.6% and 8.2%, respectively, *P* ≤ 0.0001) A1c levels. For Other/Multi-racials with self-reported prediabetes, there was a significant reduction of those with a normoglycemic (<5.7%) A1c levels (79.2% and 40.3%, respectively, *P* = 0.0104) and a significant increase in those with diabetic (≥ 6.5%) (0% and 38.8%, respectively, *P* = 0.0067) A1c levels (Table 4).

**Self-reported: Non-prediabetes**

Descriptive analysis: The total weighted sample of NHANES 2007-2008 (*n* = 164914962) and NHANES 2009-2010 (*n* = 161120813) comprised all participants aged 20 years and older with non-prediabetes based on self-report. For this group, over 19% of the participants

in the NHANES 2007-2008 and NHANES 2009-2010 surveys had prediabetes based on A1c levels (Table 4). For all five ethnic groups, based on A1c levels, there was an increase in those with prediabetes (5.7%-6.4%) and a reduction in diabetes (≥ 6.5%), except for Mexican Americans (Table 5).

$\chi^2$ : There was significant differences for all ethnic groups in all A1c levels in participants of the NHANES 2007-2008 and NHANES 2009-2010 surveys (Table 5).

Overall, for those in both categories – self-reported prediabetes and self-reported non-prediabetes – there was a combined total of 2.1% (3635002) in NHANES 2007-2008 and 2.3% (3735170) in NHANES 2009-2010 with undiagnosed diabetes.

**DISCUSSION**

**Self-reported prediabetes**

The current study demonstrates a shift in those with self-reported prediabetes in NHANES 2007-2008 (6566166) and NHANES 2009-2010 (7569333). In this sample finding, there was a 10.9% decrease in normoglycemic A1c levels (reversed prediabetes), a 3.5% increase in

prediabetic and a 7.4% increase in diabetic A1c levels from NHANES 2007-2008 to NHANES 2009-2010. This shows an inverse change, indicating that the two NHANES surveys are 2-year apart, potentially those who once had reversed prediabetes in NHANES 2007-2008 developed either prediabetes or diabetes in NHANES 2009-2010. Studies have shown that most with prediabetes will develop diabetes within 10 years unless they make lifestyle changes (losing 5%-7% of their body weight and increase physical activity)<sup>[10]</sup>.

When examining different ethnic groups, the current study demonstrates that the number of Mexican Americans, Other Hispanics and Non-Hispanic Whites with self-reported prediabetes and a prediabetic A1c level either decreased or remained the same, but Non-Hispanic Blacks nearly doubled (32.6% and 63.8%, respectively). There is no published data on the number of Non-Hispanic Blacks with prediabetes, but it is known that Non-Hispanic Black adults are twice as likely than Non-Hispanic Whites to have diagnosed diabetes<sup>[16]</sup>. The findings of this study indicate that it is the same for Non-Hispanic Blacks with prediabetes.

Given the paucity of prediabetes data on different ethnic groups, this current study showed a shift from prediabetic and diabetic A1c levels from NHANES 2007-2008 and NHANES 2009-2010. First, there was a reduction of prediabetic A1c levels (10.3%) in Other Hispanics, but an increase with diabetic (5.2%) A1c levels. There was a reduction in normoglycemic (8.9%) and prediabetic (0.7%) A1c levels in Non-Hispanic Whites, but an increase in diabetic (9.6%) A1c levels. Finally, there was a reduction in normoglycemic (38.9%) A1c levels, which was directly reflected in an increase in diabetic (38.9%) A1c levels in Other/Multi-racials. These findings show that, among these three ethnic groups with self-reported prediabetes who had either normoglycemic or prediabetic A1c levels, more than half of them actually had diabetic A1c levels, showing they were undiagnosed and unaware of having diabetes. These findings support the CDC study, which reports that 90%-95% of all cases of diabetes are of people who once had prediabetes<sup>[4]</sup>, and who are at high risk of developing diabetes complications, resulting from a lack of treatment and management of the disease.

### Non-prediabetes

The current study demonstrates that of the total weighted sample in NHANES 2007-2008 (164914962) and NHANES 2009-2010 (161120813), there was a rise in the percentage of those participants within those timeframes with undiagnosed prediabetic (19.9% and 22.4%, respectively) A1c levels, but a slight reduction of those with undiagnosed diabetic (1.9% and 1.6%, respectively) A1c levels. More than 20% of these participants either had undiagnosed prediabetes or diabetes (Table 4). For ethnic groups with non-prediabetes, there was a rise in the combined percentages of both undiagnosed prediabetes and diabetes in those from NHANES 2007-2008

to NHANES 2009-2010. The largest increase was among Mexican Americans: in NHANES 2007-2008, there was a combined 17.1% of Mexican Americans with undiagnosed prediabetes and diabetes. In NHANES 2009-2010, it had increased to 27% (Table 5). But when combining undiagnosed prediabetes and undiagnosed diabetes among both groups (self-reported prediabetes and self-reported non-prediabetes), the percentage of those with undiagnosed prediabetes and diabetes is 22.9%. These findings show that these groups who are undiagnosed are also unaware of their condition and risks, making early identification and improved awareness of prediabetes and diabetes critical first steps to motivate and encourage them to make healthy lifestyle changes.

Overall, from 2007-2009, the number of people who self-report their prediabetes has grown. There have also been a growing number of people with undiagnosed diabetes who are unaware of their condition. Those with undiagnosed diabetes had A1c levels indicating they had the disease, yet self-reported not having prediabetes or diabetes. Given that diabetes is a growing epidemic, it is essential and vital that awareness on the importance of those with risk for developing diabetes occurs early. It also is important for healthcare providers to actively screen those at risk for diabetes. These growing numbers of people with undiagnosed prediabetes and diabetes is a grave concern, given the lack of preventative care, treatment (*e.g.*, medications) and of overall healthcare services (*i.e.*, receiving a glucometer) they are not receiving and initiating. Hence, it is especially important that prediabetes trends and differences in the prevalence of reversed, diagnosed and undiagnosed prediabetes, and undiagnosed diabetes, including the prevalence of ethnicity among these groups be monitored.

### Implications for Practice

There are evidenced-based recommendations, which were developed by the American College of Endocrinology (ACE) and the American Association of Clinical Endocrinologists (AACE)<sup>[17]</sup>. In addition, the AACE has an algorithm specific for prediabetes management<sup>[18]</sup>. These recommendations and algorithm were developed for all healthcare providers, and can be used in any healthcare setting, with the goal of maintaining normal blood pressure, lipid and glucose control. The recommendations emphasize that people with prediabetes should follow the same management goals as those with diabetes. They provide treatment modalities for people with prediabetes which can be provided in any setting. For example, in an outpatient setting, the healthcare provider, during assessment, has the opportunity to gather the patient's prediabetes health history (diabetes family history, overweight or obese status or sedentary lifestyle); develop with the patient an individualized, patient-centered prediabetes plan of care; teach patients that prediabetes is a risk factor for diabetes; teach normal A1c, blood pressure and lipid levels; and evaluate the patient's adherence to the treatment plan. Also, the provider would include in the teach-

ing plan the importance of adherence to the medication regimen (e.g., Metformin), following a low-carbohydrate nutritional diet, exercising, monitoring blood glucose, and appropriately using healthcare services to prevent, detect and treat diabetes and its complications. Another important setting where healthcare providers could utilize the ACE and AACE recommendations and algorithm would be in the community. Healthcare providers could develop and initiate collaborative awareness campaigns on prediabetes, diabetes, its complications and risk factors associated with diabetes. In the awareness campaigns, healthcare providers could use these recommendations to teach about differences between prediabetes and diabetes, clinical risks of poor prediabetes management, treatment modalities and goals. Community providers could partner with senior centers and faith-based organizations, and conduct community events reaching out to different high-risk communities. Because these recommendations were developed to improve prediabetes outcomes and reduce the development of diabetes and its related complications, healthcare providers in any healthcare setting can utilize and implement these recommendations.

Healthcare providers need to ensure that other providers are aware of the ACE and AACE prediabetes recommendations and algorithm. They need to advocate that such evidenced tools are available and should be used in practice for early screening and treatment of prediabetes. Examples of venues healthcare providers can use to educate and advocate the use of these recommendations and algorithm, include speaking about it at educational forums on prediabetes or diabetes; becoming members of diabetes organizations, networking with others to promote and participate in projects or workshops on prediabetes or diabetes, conducting research and writing publications on prediabetes and the usage of these recommendations and algorithm in practice, and by becoming a board member of an association to contribute, shape and change policies related to improving prediabetes care.

### Limitations

There are several limitations to this current study. First, “being told” or not “being told they have prediabetes”, and “diabetes” was based on self-report. Verification of a participant’s diagnosis (i.e., having or not having prediabetes or diabetes) by a primary care provider (PCP) could not be conducted since NHANES does not collect such data. Second, NHANES data does not include participant’s prediabetes or diabetes follow-up practices with their PCP, and could not be examined in this current study. Third, although NHANES surveys have collected Oral Glucose-Tolerance Test (OGTT) since 2005, it is not regularly collected on all eligible persons in the survey<sup>[19]</sup>. For example, NCHS collected OGTT on all eligible participants in the NHANES 2007-2008 survey, but not for all eligible participants, only for a subsample (i.e., a smaller sample) of the NHANES 2009-2010 survey<sup>[20]</sup>. Hence, OGTT data was not used in this current study,

Fourth, although FPG and A1c were collected in both NHANES 2007-2008 and NHANES 2009-2010, since A1c is the most common clinical tool used to measure glucose levels, A1c was the preferred measurement used in this current study. Fifth, given the lag time of large data sets, between data collection and data availability<sup>[21]</sup>, the most current data sets available in 2013 with the variables needed for data analysis were NHANES 2007-2008 and NHANES 2009-2010. For that reason, they were used in this current study. Despite these limitations, the data provide vital information on normoglycemic, prediabetic and diabetic A1c prevalence in those with self-reported prediabetes, in addition to prediabetic and diabetic A1c levels in those with self-reported non-prediabetes (i.e., self-report indicates has not ever had prediabetes).

## COMMENTS

### Background

Studies have shown that the majority of people with prediabetes do not know they have it and are unaware of their risk for diabetes. As the number of those with prediabetes increases so is the number of those at risk for developing diabetes. Hence, it is essential that healthcare providers increase awareness of prediabetes among the public, and initiate early detection and treatment of prediabetes in order to prevent the development of diabetes and its complications.

### Research frontiers

National Health and Nutritional Examination Survey (NHANES) 2007-2008 and NHANES 2009-2010 data among five specific ethnic/racial groups (Non-Hispanic Whites, Non-Hispanic Blacks, Mexican Americans, Other Hispanics, and Other/Multiracial) showed the prevalence of normoglycemic, prediabetic and diabetic A1c levels in those with prediabetes; and prediabetic and diabetic A1c levels in those with non-prediabetes. In addition, the study showed those with prediabetes, undiagnosed prediabetes and undiagnosed diabetes.

### Innovations and breakthroughs

This study demonstrated that undiagnosed/unaware diabetes from NHANES 2007-2008 to NHANES 2009-2010 data showed an increase in the percentage of those with self-reported prediabetes and diabetic A1c levels, also known as undiagnosed or unaware of having diabetes. Of the five different ethnic groups, Other Hispanic, Non-Hispanic Whites, and Other/Multi-racial groups who self-reported prediabetes had diabetic A1c levels. For all ethnic groups who self-reported not having prediabetes had prediabetic A1c levels. These groups of participants show a high rate of people who are either undiagnosed or unaware of having prediabetes or diabetes.

### Applications

Results of this study show the importance of awareness and outreach and the need for more community-based education.

### Peer review

Differences among five ethnic groups were examined and provide a unique insight since the literature has focused on differences in the prevalence of diabetes rather than prediabetes. Given that the latest statistics showed that 79 million people have prediabetes ways to address the lack of education in the community and close the gap are essential in order to decrease the number of people unaware of having prediabetes or diabetes.

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## Vildagliptin vs sulfonylurea in Indian Muslim diabetes patients fasting during Ramadan

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ventional, open-label, observational study. Incidence of hypoglycemic events (HEs), adverse events, and changes in glycosylated hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), fasting plasma glucose, postprandial plasma glucose and body weight were measured pre- and post-Ramadan.

**RESULTS:** Totally, 97 patients were recruited and all completed the study (vildagliptin group,  $n = 55$ ; sulfonylurea group,  $n = 42$ ). HEs were reported in low frequencies in both the vildagliptin and the sulfonylurea groups [0 vs 2 (4.8%) patients, respectively]. Interestingly, HbA<sub>1c</sub> reduced by -0.43% (-4.71 mmol/mol) in the vildagliptin group [8.75% (72.10 mmol/mol) to 8.32% (67.38 mmol/mol),  $P = 0.009$ ] while in the sulfonylurea group there was a small increase by 0.01% [0.08 mmol/mol; 8.64% (70.92 mmol/mol) to 8.65% (71.00 mmol/mol),  $P = 0.958$ ]. Higher percentage of vildagliptin-treated patients achieved HbA<sub>1c</sub> < 7.0% (< 53 mmol/mol) compared with sulfonylurea (16.4% vs 4.8%). Mean decrease in the body weight was 1.2 kg and 0.03 kg, respectively ( $P < 0.001$ ). Both treatment groups were well tolerated during Ramadan.

**CONCLUSION:** Vildagliptin is an attractive treatment option for Indian patients with type 2 diabetes mellitus who are fasting during Ramadan.

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**Key words:** Dipeptidyl-peptidase 4 inhibitors; Fasting hypoglycemia; Sulfonylurea; Type 2 diabetes mellitus; Vildagliptin

**Core tip:** Management of glycemic control in diabetes patients fasting during Ramadan has been recognized as a critically important health challenge worldwide. India has the world's second largest diabetes population and caters large Muslim community; however, there is limited data available exploring the effect of treatments

### Abstract

**AIM:** To compare the use of vildagliptin and sulfonylurea with or without metformin in Indian Muslim patients with type 2 diabetes mellitus, fasting during Ramadan.

**METHODS:** This was a 4-wk, multicenter, non-inter-

in these fasting diabetes patients. This non-interventional, multicenter, double-arm study compared the effect of vildagliptin with sulfonylureas on hypoglycemic events, HbA<sub>1c</sub>, blood glucose levels, and response rate in 97 fasting diabetic patients during Ramadan in real-world setting. Vildagliptin appears to be an attractive treatment option for diabetes patients fasting during Ramadan.

Shete A, Shaikh A, Nayeem KJ, Rodrigues L, Ali MSS, Shah P, Khanna R, Majid S, Rasheed SA, Shaikh S, Rahman T. Vildagliptin vs sulfonylurea in Indian Muslim diabetes patients fasting during Ramadan. *World J Diabetes* 2013; 4(6): 358-364 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/358.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.358>

## INTRODUCTION

Worldwide, about 40-50 million Muslim patients with type 2 diabetes mellitus observe fasting during the holy month of Ramadan<sup>[1]</sup>. India has the world's largest diabetes population, with an estimated 51 million affected individuals<sup>[2]</sup>. The prevalence of diabetes is rising by 10% annually in countries with large Muslim populations, similar to the rates noted in Western countries, as an upshot of urbanization and socioeconomic development<sup>[3]</sup>.

Muslims, on average, fast for 12 h per day during Ramadan, which varies from country to country and from season to season and lasts for up to 30 d<sup>[4]</sup>. Although Islamic rules exempt patients with illnesses from fasting, almost 79% of patients with type 2 diabetes mellitus prefer to fast; therefore, it is essential to make fasting as safe as possible<sup>[5]</sup>. As the fast is absolute in nature, that is, without water and food from dawn to dusk, hypoglycemia presents a serious risk<sup>[1]</sup>. Prolonged fasting in the absence of adequate insulin can cause excessive gluconeogenesis leading to hyperglycemia. There is also a risk of severe post-prandial hyperglycemia in those who tend to overeat after breaking the fast<sup>[5]</sup>. The Epidemiology of Diabetes and Ramadan study conducted in 13 Islamic countries reported that in patients with type 2 diabetes mellitus, fasting during Ramadan increased the risk of severe hypoglycemia by 7.5-fold and severe hyperglycemia by 5-fold. During Ramadan, up to 2% of fasting patients with type 2 diabetes mellitus may experience at least one episode of severe hypoglycemia requiring hospitalization<sup>[5]</sup>.

Improved diabetes management during Ramadan can be achieved by appropriate treatment adjustments such as timing and dosage of the drug<sup>[5]</sup>. The American Diabetes Association emphasizes the importance of monitoring blood glucose levels and providing nutritional advice and Ramadan-focused structured education in patients with type 2 diabetes mellitus<sup>[5]</sup>.

Improved glycemic control without hypoglycemia and weight gain is the key goal in the management of diabetes; however, achieving this goal is challenging in patients

with diabetes, especially who are fasting. Therapies that affect the incretin system like dipeptidyl-peptidase-4 (DPP-4) inhibitors, which could maintain glycemic control in a glucose-dependent manner, might provide a safe alternative therapeutic option during Ramadan<sup>[5]</sup>. Vildagliptin, a potent and selective oral DPP-4 inhibitor, improves glycemic control in patients with type 2 diabetes mellitus by improving both  $\alpha$ - and  $\beta$ -cell responsiveness to glucose<sup>[6-8]</sup>. A long-term randomized clinical study with vildagliptin demonstrated significantly lower hypoglycemic risk compared with sulfonylureas in patients with type 2 diabetes mellitus<sup>[9,10]</sup>. A real-world observational and non-interventional study, VECTOR, conducted in United Kingdom Muslim patients with type 2 diabetes mellitus fasting during Ramadan demonstrated no hypoglycemic events (HEs) and better glycemic control with vildagliptin compared with gliclazide<sup>[11]</sup>. In a similar study by Devendra *et al*<sup>[12]</sup> conducted in North West London, both vildagliptin and gliclazide were associated with similar reductions in mean glycosylated haemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>).

Although there is data available for vildagliptin in fasting Ramadan patients, the present study was conducted to assess whether similar efficacy and safety results are also seen in Indian patients with type 2 diabetes mellitus fasting during Ramadan.

## MATERIALS AND METHODS

### Study design

This was an 8- to 10-wk, multicenter, non-interventional, prospective, open-label, observational study conducted across 10 centers in India in 2010. Patients with type 2 diabetes mellitus, aged 18-80 years, fasting during Ramadan and taking vildagliptin with or without metformin (hereafter called the vildagliptin group) or sulfonylureas with or without metformin (hereafter called the sulfonylurea group) were included in the study. Patients were given Ramadan-focused advice about diabetes management. Dosages were given according to the approved package labels. Patients receiving insulin and/or having contraindications as described in the summary of product characteristics for vildagliptin (with or without metformin; Galvus<sup>®</sup> or GalvusMet<sup>®</sup>) (pregnancy and hypersensitivity) were not recruited in the study.

Following a screening visit during the 2- to 3-wk pre-Ramadan period, eligible patients were observed for 4 wk (29 d) during the Ramadan period, followed by a second visit within 10 d post-Ramadan.

This study was conducted and reported in accordance with the International Conference on Harmonization for Good Clinical Practice, with applicable local regulations and with the ethical principles laid down in the Declaration of Helsinki. Protocol and any amendments were reviewed by the Independent Ethics Committee. Written informed consent was obtained from each patient or by a legally acceptable representative before inclusion in the study.

**Table 1** Demographics and baseline characteristics

	Vildagliptin group (n = 55)	Sulfonylurea group (n = 42)
Age (yr)	51.0 ± 8.8	50.9 ± 9.1
≥ 65 yr	4 (7.3)	4 (9.5)
Weight, kg	69.1 ± 10.9	67.4 ± 10.0
Height, cm	159.7 ± 8.1	158.4 ± 9.8
HbA <sub>1c</sub> , % (mmol/mol)	8.75 ± 1.27 (72.10 ± 13.90)	8.64 ± 1.57 (70.92 ± 17.14)
HbA <sub>1c</sub> < 7.0% (< 53.0 mmol/mol)	3 (5.5)	6 (14.3)
FPG, mg/dL	140.2 ± 42.0	162.1 ± 48.2
PPG, mg/dL	212.2 ± 52.2	220.6 ± 54.0
Patients receiving metformin, n (%)	38 (69.1)	30 (71.4)
Mean doses of sulfonylureas, mg/d		
Glibenclamide (n = 7)	-	10
Gliclazide (n = 10)	-	106
Glimepiride (n = 23)	-	4.2
Glipizide (n = 2)	-	10

Data are shown as mean ± SD or n (%), unless otherwise stated. Vildagliptin group: Patients receiving vildagliptin ± metformin; Sulfonylurea group: Patients receiving sulfonylurea ± metformin; FPG: Fasting plasma glucose; PPG: Postprandial plasma glucose.

### Study assessments

The primary assessment was incidence of HEs during the study period. Patients were provided with a questionnaire for self-assessment of HEs based on symptoms, which were confirmed by blood glucose levels < 70 mg/dL either self-measured with a glucometer (Accu-Chek Active®, Roche) and recorded in patients' diaries or measured at any laboratory. Secondary assessments included mean changes in HbA<sub>1c</sub>, body weight, fasting plasma glucose (FPG) and postprandial plasma glucose (PPG, glucose levels following breakfast) from pre-Ramadan (baseline) to post-Ramadan (endpoint) period. The proportion of patients reaching the HbA<sub>1c</sub> goal < 7.0% (< 53 mmol/mol) during the study period was also determined. Safety assessments included monitoring and recording of all adverse events (AEs), serious AEs (SAEs) and laboratory tests.

### Sample size and power

A sample size of 72 patients (minimum 36 patients in each treatment group) with type 2 diabetes mellitus was estimated to detect the difference between the two groups at 95%CI and a study power of 80%. This was based on data from previous international clinical trials, which reported that one-third of the patients in the sulfonylurea group and 5% of the patients in the vildagliptin group developed hypoglycemia during Ramadan fasting.

### Statistical analysis

The null hypothesis, which was proportion of patients experiencing at least one HE was the same in each cohort, was tested using a 2-group Fisher's exact test. The proportion of patients experiencing an AE was described in terms of their frequency and percentage for each event (the basis of percentage being the number of patients who provided data). Descriptive statistics were used to analyze quantitative variables such as change in

body weight, FPG, PPG and HbA<sub>1c</sub> levels. Paired *t*-test was performed to assess the statistical significance of the differences between pre- and post-Ramadan periods and unpaired *t* test for the between-group differences at 95%CI.

## RESULTS

### Study patients

A total of 97 patients (55 in the vildagliptin group and 42 in the sulfonylurea group) were enrolled and all patients (100%) completed the study within the stipulated 10 d period post Ramadan. All patients observed fasting for a period of 4 wk (29 d). The demographics and baseline characteristics are summarized in Table 1.

### Hypoglycemic events during the fasting period

HEs were reported in low frequencies in both the vildagliptin group and the sulfonylurea group [0 *vs* 2 (4.8%) patients, respectively]. In the sulfonylurea group one HE was reported by patient who was taking glipizide 5 mg twice a day and the other HE was reported by patient who was taking glibenclamide 5 mg twice a day during the study period. The difference in the proportion of patients who experienced at least one HE during the study period was not significant (*P* = 0.104) between the treatment groups.

### Reduction in HbA<sub>1c</sub>

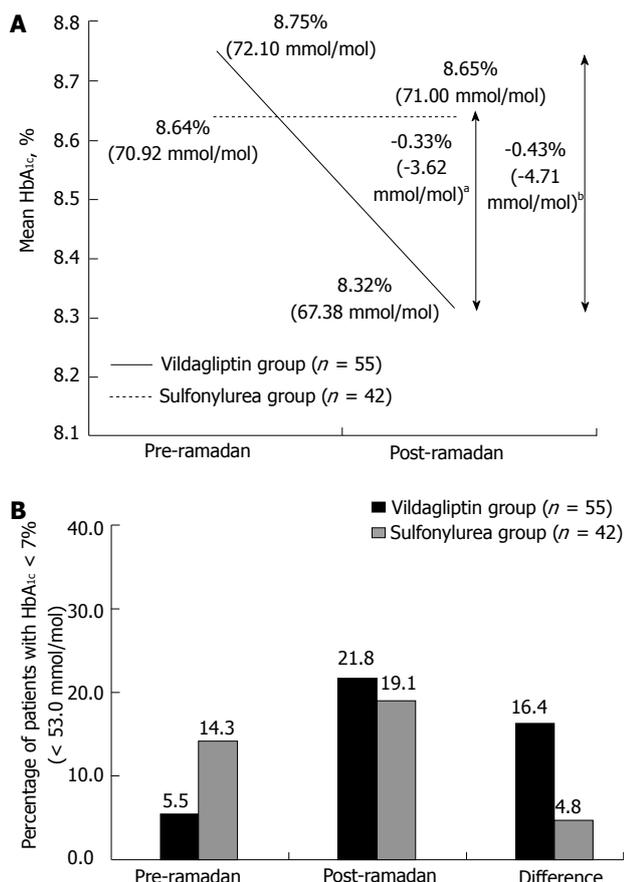
The reduction in HbA<sub>1c</sub> from pre- to post-Ramadan was statistically significant in the vildagliptin group [0.43% (4.71 mmol/mol), *P* = 0.009], whereas there was a negligible increase in HbA<sub>1c</sub> in the sulfonylurea group [0.01% (0.08 mmol/mol), *P* = 0.958]. The mean between-group difference for the change in HbA<sub>1c</sub> from pre- to post-Ramadan was statistically significant (*P* < 0.05) in favor of the vildagliptin group (Figure 1A). In patients who had HbA<sub>1c</sub> ≥ 7.0% (≥ 53 mmol/mol) at baseline, nine patients (16.4%) achieved the target HbA<sub>1c</sub> < 7.0% (< 53 mmol/mol) in the vildagliptin group compared with only 2 patients (4.8%) in the sulfonylurea group (*P* = 0.055) (Figure 1B).

### Reduction in body weight

The vildagliptin group showed a significant reduction in mean body weight (*P* < 0.001), whereas the sulfonylurea group had negligible mean weight reduction from pre- to post-Ramadan period. The between-group difference for mean body weight reduction was statistically significant (1.2 kg *vs* 0.03 kg; *P* < 0.001) in favor of vildagliptin (Figure 2).

### Reduction in blood glucose levels

Mean FPG and PPG decreased significantly in both the groups from pre- to post-Ramadan period. The reduction in mean FPG was 14.7 mg/dL (pre-Ramadan *vs* post-Ramadan; 140.2 mg/dL *vs* 125.5 mg/dL; *P* = 0.015) and 20.0 mg/dL (162.1 mg/dL *vs* 142.1 mg/dL; *P* = 0.015) in the vildagliptin and sulfonylurea groups,



**Figure 1** Glycosylated hemoglobin A<sub>1c</sub> changes from pre- to post-Ramadan period (A) and percentage of patients who achieved target glycosylated hemoglobin A<sub>1c</sub> < 7.0% (B). <sup>a</sup>P < 0.05, <sup>b</sup>P = 0.009. Vildagliptin group: Patients receiving vildagliptin ± metformin; Sulfonylurea group: Patients receiving sulfonylurea ± metformin; HbA<sub>1c</sub>: Glycosylated hemoglobin A<sub>1c</sub>.

respectively. Similarly, the drop in mean PPG was 26.2 mg/dL (212.2 mg/dL vs 186.0 mg/dL; P = 0.001) and 33.1 mg/dL (220.6 mg/dL vs 187.5 mg/dL; P = 0.001) in the vildagliptin and sulfonylurea groups, respectively. The between-group differences for reductions in FPG and PPG were not statistically significant.

### Safety assessments

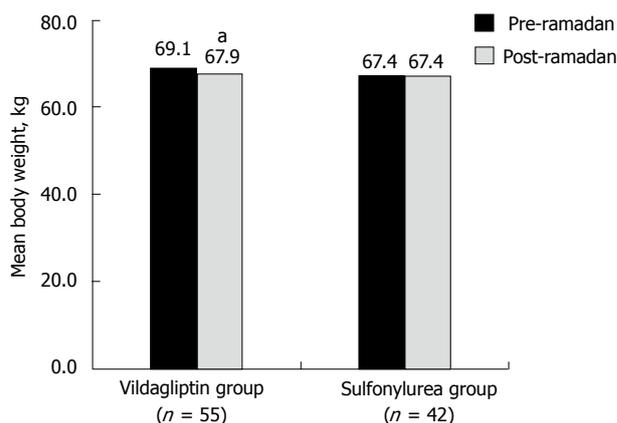
The overall incidence of AEs was 5.5% in the vildagliptin group and 9.5% in the sulfonylurea group (largely driven by the 2 HEs). The AEs reported in the vildagliptin group and the sulfonylurea group are listed in Table 2. No SAEs, drug-related AEs or discontinuations due to AEs were reported in either group.

## DISCUSSION

This is the first study to compare the efficacy and safety of vildagliptin with sulfonylureas in fasting Indian patients with type 2 diabetes during Ramadan. This 8-10 wk, real-world, observational study in Indian Muslim patients with type 2 diabetes mellitus, fasting during Ramadan reported no significant differences in HEs between the vildagliptin and sulfonylurea groups, with only 0 and

**Table 2** Safety and tolerability of vildagliptin and sulfonylurea (n (%))

Adverse events	Vildagliptin group (n = 55)	Sulfonylurea group (n = 42)
Number of patients with adverse event(s)	3 (5.5)	4 (9.5)
Palpitation	2 (3.6)	1 (2.4)
Gastrointestinal disturbances	2 (3.6)	0 (0.0)
Hypoglycemia	0 (0.0)	2 (4.8)
Headache	0 (0.0)	1 (2.4)
Nausea	0 (0.0)	1 (2.4)



**Figure 2** Mean body weight at pre- and post-Ramadan period. <sup>a</sup>P < 0.01. Vildagliptin group: Patients receiving vildagliptin ± metformin; Sulfonylurea group: Patients receiving sulfonylurea ± metformin.

2 HEs in those two groups, respectively. The vildagliptin group showed greater reductions in HbA<sub>1c</sub> compared with the sulfonylurea group, although the mean FPG and PPG levels decreased significantly in both the groups. The percentage of patients reaching target HbA<sub>1c</sub> < 7.0% (< 53 mmol/mol) was 3-fold higher in the vildagliptin group compared with the sulfonylurea group. The vildagliptin group also showed a significant but modest reduction in the body weight compared with the sulfonylurea group. The overall incidence of AEs was lower in the vildagliptin group compared with the sulfonylurea group, and there were no drug-related SAEs or discontinuations due to AEs in either group.

Achieving glycemic control without hypoglycemia during fasting, especially during Ramadan, which spans for a period of 4 wk, is challenging in patients with type 2 diabetes mellitus. Incretin therapies like DPP-4 inhibitors are potentially safe during Ramadan<sup>[5]</sup>. Vildagliptin, a potent and selective oral DPP-4 inhibitor, has been shown to improve insulin secretion in response to glucose levels<sup>[13]</sup> and suppress inappropriate glucagon response to glucose<sup>[14,15]</sup>, thus maintaining glycemic control in a glucose-dependent manner. In the present study, vildagliptin reduced HbA<sub>1c</sub> without any incidence of HE, which indicates a regulated glycemic control during Ramadan fasting, while there was no mean HbA<sub>1c</sub> drop with a few HEs in the sulfonylurea group. The HbA<sub>1c</sub> differences may be driven by the meals and/or compliance to medication,

which were not assessed. However, the results need a cautious interpretation, given that Ramadan lasted only a month and the fact that at least 3 mo may be required for a given change in blood glucose levels to translate to a stable and maximal HbA<sub>1c</sub> change is likely to misestimate the true lowering of average blood glucose levels during Ramadan.

The low incidence of HEs with sulfonylureas may be explained by the high baseline HbA<sub>1c</sub> of > 8.5% where hypoglycemic rates are lower and by the fear of hypoglycemia associated with sulfonylureas leading to non-compliance. For instance, the relatively low number of HEs in the sulfonylurea group is consistent with the observation in an earlier study in which the investigators chose not to escalate the dose in the first 16 wk of the study to avoid hypoglycemia<sup>[10]</sup>. This may also explain the lack of decrease in the mean HbA<sub>1c</sub> in the sulfonylurea group over 10 wk in this study. Failure to lower HbA<sub>1c</sub> is not likely explained by over-eating, due to the specific pattern of food intake including Ramadan fasting from dawn to dusk and feasting before and after the fast, since there was no increase in weight. The lack of weight gain in the sulfonylurea group appears to rule out defensive eating as an alternative mechanism to explain the low incidence of HEs.

Efficacy and safety of vildagliptin as monotherapy or in combination with other oral anti-diabetic drugs has been reported extensively<sup>[6,17-25]</sup>. A long-term study with vildagliptin as add-on to metformin compared with glimepiride demonstrated a lower proportion of patients experiencing HEs over 1 year (1.7% *vs* 16.2%) and 2 years (2.3% *vs* 18.2%) in a randomized clinical trial in patients with type 2 diabetes mellitus<sup>[9,10]</sup>. In Muslim patients with type 2 diabetes mellitus who were fasting during Ramadan, in whom hypoglycemia remains a major risk, vildagliptin, compared with sulfonylureas, showed no or lower incidence of HEs<sup>[11,12]</sup>. In one observational study, compared with gliclazide, vildagliptin demonstrated a significantly lower number of HEs (2 *vs* 24) and lower proportion of patients experiencing HEs (7.7% *vs* 61.5%;  $P < 0.001$ ) in Muslim type 2 diabetes mellitus patients fasting during Ramadan. Moreover, shifting from sulfonylurea to vildagliptin was associated with a reduction in HEs during Ramadan, while continuation with sulfonylurea treatment showed an increase in the number of HEs<sup>[12]</sup>.

Furthermore, in the VECTOR study conducted in United Kingdom Muslim patients with type 2 diabetes mellitus fasting during Ramadan, vildagliptin demonstrated significantly better glycemic control than gliclazide (mean difference between treatments for change in HbA<sub>1c</sub> was 0.5%,  $P < 0.0262$ ) and accompanied with no reports of HEs or severe HEs, while 34 HEs in 15 patients and one severe HE (grade 2) were reported with gliclazide<sup>[11]</sup>. The incidence of hypoglycemia with vildagliptin in the present study is in line with the VECTOR study, while sulfonylurea is associated with a lower hypoglycemic incidence than in the VECTOR study. These differences could be partly explained by the higher baseline of > 8.5%

in both treatment arms of this study and the fact that our study did not specifically look at treatment compliance during Ramadan.

Vildagliptin is known to maintain weight neutrality<sup>[26,27]</sup>, whereas a trend towards weight gain is known with sulfonylurea<sup>[11]</sup>. However, in this study, a reduction in body weight with vildagliptin was observed in Muslim patients with type 2 diabetes fasting during Ramadan, whereas there was a negligible change with sulfonylurea. Although not investigated, a lesser food or carbohydrate intake associated with fasting and physical activity could be a reason for differential effect of both treatments on weight compared with earlier studies. Noncompliance to treatment could be another possible reason for weight neutrality in the sulphonylurea group in this study.

A few limitations of this study include a small sample size, lack of data on treatment adherence, diet, eating pattern and exercise. Although the treatment adherence was not measured in this study, the VECTOR study showed a significantly better treatment adherence for vildagliptin compared with sulfonylurea, most likely due to the better tolerability and less fear of hypoglycemia<sup>[11]</sup>. Overall, vildagliptin compared with sulphonylurea showed an improvement in the glycemic control with no accompanied HEs in this study. This might be the reason for not attaining mean HbA<sub>1c</sub> drop in the sulfonylurea group over 10 wk in this study.

However, despite all the limitations, there appears to be a trend suggestive of good glycemic control with vildagliptin. This effect is particularly meaningful when looked in the context of no HEs (and at least no weight gain) seen with vildagliptin during fasting. These effects need to be confirmed by larger, randomized, interventional trials assessing the relative value of a vildagliptin compared with sulfonylurea in fasting type 2 diabetes patients during Ramadan.

Vildagliptin was not associated with hypoglycemia and tended towards better glycemic control than sulfonylurea. Vildagliptin appears to be an attractive treatment option for Indian patients with type 2 diabetes mellitus who are fasting during Ramadan.

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## COMMENTS

### Background

Muslims, on average, fast for 12 h per day during Ramadan, which varies from country to country and from season to season and lasts for up to 30 d. Almost 79% of patients with type 2 diabetes mellitus prefer to fast; therefore, it is essential to make fasting as safe as possible. Hypoglycemia presents a serious

risk in these patients and it is necessary to test treatment options in this important population group.

### Research frontiers

Management of glycemic control in diabetes patients fasting during Ramadan has been recognized as a critically important health challenge worldwide. Therapies that affect the incretin system like dipeptidyl-peptidase-4 inhibitors, which could maintain glycemic control in a glucose-dependent manner, might provide a safe alternative therapeutic option during Ramadan. India has the world's second largest diabetes population and caters large Muslim community; however, there is limited data available exploring the effect of treatments in these fasting diabetes patients.

### Innovations and breakthroughs

The current study assesses the relative value for vildagliptin compared to sulfonylurea in fasting type 2 diabetes Muslim patients in a real-world Indian setting and adds useful evidence to the currently limited evidence in this setting.

### Applications

This study could guide the physicians in selecting suitable antidiabetic agent to manage glycemic control in Indian Muslims patients with diabetes who tend to fast from dawn to dusk during the month of Ramadan.

### Terminology

Hypoglycemia was the primary assessment during the study period. Patients were provided with a questionnaire for self-assessment of hypoglycemic event based on symptoms, which were confirmed by blood glucose levels < 70 mg/dL either self-measured with a glucometer (Accu-Chek Active®, Roche) and recorded in patients' diaries or measured at any laboratory and were also assessed by the treating physicians.

### Peer review

The primary aim of this study is to compare the effect of vildagliptin and sulfonylureas on the incidence of hypoglycemia events, adverse events, glycemic control, and body mass in Indian Muslim patients during Ramadan. The authors should raise the issue that the use of glycosylated haemoglobin A<sub>1c</sub> levels as a means to assess glycemic control is likely to misestimate the true lowering of average blood glucose levels during Ramadan.

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## Effect of hypolipidemic treatment on glycemic profile in patients with mixed dyslipidemia

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### Abstract

**AIM:** To assess the effect of different hypolipidemic treatment strategies on glycemic profile in mixed dyslipidemia patients.

**METHODS:** This is a prespecified analysis of a prospective, randomized, open-label, blinded end point (PROBE) study (ClinicalTrials.gov identifier: NCT01010516). Patients ( $n = 100$ ) with mixed dyslipidemia on a standard statin dose who had not achieved lipid targets were randomized to switch to the highest dose of rosuvastatin (40 mg/d) or to add-on-statin extended release nicotinic acid (ER-NA)/laropiprant (LRPT) or to add-on-statin micronised fenofibrate for a total of 3 mo. Fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), homeostasis model assessment of insulin resistance (HOMA-IR) index and lipid profile were evaluated at baseline and 3 mo after treatment intervention.

**RESULTS:** FPG increased in add-on ER-NA/LRPT and rosuvastatin monotherapy groups by 9.7% and 4.4%,

respectively ( $P < 0.01$  between the 2 groups and compared with baseline), while it did not significantly change in the add-on fenofibrate group. Similarly, HbA1c increased by 0.3% in add-on ER-NA/LRPT group and by 0.2% in the rosuvastatin monotherapy group ( $P < 0.01$  for all comparisons *vs* baseline and for the comparison between the 2 groups), while no significant change was reported in the add-on fenofibrate group. HOMA-IR increased by 65% in add-on ER-NA/LRPT and by 14% in rosuvastatin monotherapy group, while it decreased by 6% in the add-on fenofibrate group ( $P < 0.01$  *vs* baseline and for all comparisons among the groups). Non-HDL-C decreased in all groups (by 23.7%, 24.7% and 7% in the rosuvastatin, ER-NA/LRPT and fenofibrate group, respectively,  $P < 0.01$  for all *vs* baseline and  $P < 0.01$  for all *vs* with fenofibrate group).

**CONCLUSION:** Both addition of ER-NA/LRPT and switch to the highest dose of rosuvastatin deteriorated glycemic profile in patients with mixed dyslipidemia, while add-on fenofibrate seems to increase insulin sensitivity.

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**Key words:** Diabetes mellitus; Fasting plasma glucose; Fenofibrate; Insulin; Nicotinic acid/laropiprant; Rosuvastatin

**Core tip:** In this study both addition of extended release nicotinic acid/laropiprant and switch to the highest dose of rosuvastatin deteriorated glycemic profile in patients with mixed dyslipidemia who were inadequately controlled with a standard statin dose. Add-on fenofibrate, on the other hand, seems to increase insulin sensitivity. Larger prospective studies should address the effect of these treatment interventions on new onset diabetes incidence and cardiovascular dis-

ease risk.

Kei A, Liberopoulos E, Elisaf M. Effect of hypolipidemic treatment on glycemic profile in patients with mixed dyslipidemia. *World J Diabetes* 2013; 4(6): 365-371 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/365.htm> DOI: <http://dx.doi.org/10.4239/wjcd.v4.i6.365>

## INTRODUCTION

As diabetes mellitus (DM) is a worldwide health problem with epidemic proportions which may lead to functional disability, vascular complications and premature death, the prevention or delay of DM development is of major clinical importance<sup>[1,2]</sup>. Lipid-lowering drugs may affect glucose metabolism in different ways. Noteworthy, a potentially diabetogenic role for statins has been suggested both from large studies and meta-analyses<sup>[3-5]</sup>. Previously, we showed that rosuvastatin may increase insulin resistance and homeostasis model assessment of insulin resistance (HOMA-IR) levels in patients with impaired fasting glucose in a dose-dependent manner and may increase the risk for new onset DM<sup>[6,7]</sup>. Similarly, nicotinic acid (NA) has been associated with both deterioration of glycemic profile and new onset DM<sup>[8]</sup>. On the other hand, fenofibrate administration has been linked with increased insulin sensitivity<sup>[9,10]</sup>.

Mixed dyslipidemia is characterized by both elevated triglyceride (TG) and low density lipoprotein cholesterol (LDL-C) levels and by reduced high-density lipoprotein cholesterol (HDL-C) levels<sup>[11]</sup>. For that, monotherapy with a conventional statin dose may not achieve all treatment targets. Currently, it remains unknown which is the best treatment strategy to address all lipid abnormalities in these patients. We recently showed that both switch to the highest dose of rosuvastatin monotherapy (40 mg) and add-on-current-statin extended release NA (ER-NA)/laropiprant (LRPT) were associated with marked reductions in non-HDL-C and LDL-C levels compared with add-on fenofibrate in patients with mixed dyslipidemia not on goal with a standard statin dose<sup>[12]</sup>. We now report the results of a prespecified analysis on the effect of these 3 treatment strategies on glycemic profile.

## MATERIALS AND METHODS

### Study population

Study details have been previously described<sup>[12]</sup>. Briefly, consecutive subjects with primary hypercholesterolemia ( $n = 100$ ) attending the Outpatient Lipid and Obesity Clinic of the University Hospital of Ioannina, Ioannina, Greece were recruited. Eligible patients were those treated for at least 3 mo with a conventional statin dose (10-40 mg simvastatin or 10-20 mg atorvastatin or 5-10 mg rosuvastatin) and their LDL-C or non-HDL-C levels were above those recommended by the National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP

III based on each patient risk factors<sup>[13]</sup>.

Subjects with TG > 500 mg/dL (5.65 mmol/L), renal disease (serum creatinine levels > 1.6 mg/dL; 141  $\mu$ mol/L), hypothyroidism [thyroid stimulating hormone (TSH) > 5 IU/mL] and liver disease [alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) levels > 3-fold upper limit of normal in 2 consecutive measurements] were excluded from the study. Patients with hypertension and/or DM were considered eligible if they were on stable medication for at least 3 mo and their blood pressure and/or glycemic profile were adequately controlled (no change in their treatment was allowed during study period).

The study had a prospective, randomized, open-label, blinded end point (PROBE) design. Patients were randomly allocated (without a wash-out phase) to open-label the highest approved dose of rosuvastatin (40 mg/d) or to add-on-current-statin treatment with ER-NA/LRPT (1000/20 mg/d for the first 4 wk, followed by 2000/40 mg/d for the next 8 wk) or to add-on-statin micronised fenofibrate (200 mg/d) for a total of 3 mo (Figure 1).

All patients were given similar dietary advice. Compliance with treatment and lifestyle habits were assessed by questionnaire and tablet count. This trial has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. All study participants gave their written informed consent prior to enrolment and the Ethics Committee of the University Hospital of Ioannina approved the study protocol. This study is registered at ClinicalTrials.gov (NCT01010516).

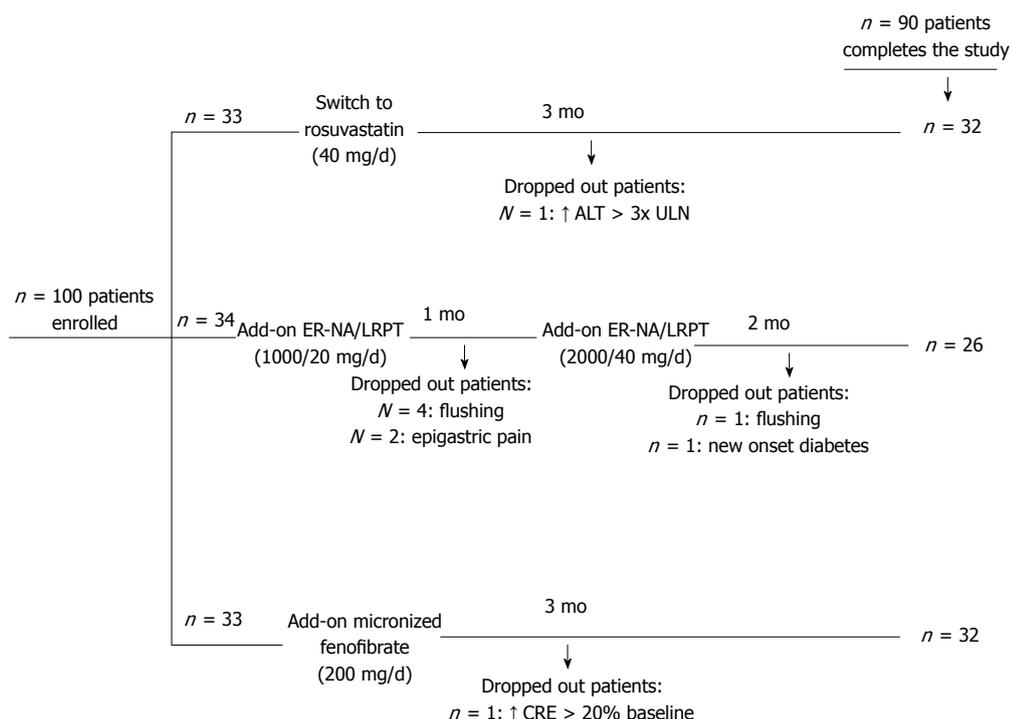
### Laboratory measurements

Blood samples for laboratory tests were obtained at baseline and 12 wk after the start of treatment after a 12-h overnight fast. Serum levels of fasting glucose were determined enzymatically in the laboratory of the University Hospital of Ioannina using an Olympus AU 600 analyzer (Olympus Diagnostica GmbH, Hamburg, Germany). Intra-assay and total coefficient variations for glucose assay were 0.7% and 1.6%, respectively. The determination of glycosylated haemoglobin (HbA1c) (expressed as percentage of the total haemoglobin concentration) was based on a latex agglutination inhibition assay (Randox Laboratories Ltd., Crumlin, United Kingdom). HbA1c values are expressed as percentage of the total haemoglobin concentration. The sensitivity of the assay is 0.25 g/dL of HbA1c and the within- and between- run precision is < 6.67% and < 4.82%, respectively. Fasting serum insulin was measured by an AxSYM insulin assay microparticle enzyme immunoassay on an AzSYM analyzer (Abbott Diagnostics, Illinois, United States). Intra-assay and total coefficient variations for insulin assay were 4.1% and 5.3%, respectively. The HOMA-IR index was calculated as follows: HOMA-IR index = fasting insulin (mU/L)  $\times$  FPG (mg/dL)/405.

### Statistical analysis

The analysis only included patients who completed the

Study participants flow diagram



**Figure 1 Study participants flow diagram.** ER-NA/LRPT: Extended release nicotinic acid/laropiprant; ALT: Alanine aminotransferase; ULN: Upper normal limit; CRE: Serum creatinine.

study as per protocol. Values are given as mean  $\pm$  SD and median (range) for parametric and non-parametric data, respectively. Continuous variables were tested for lack of normality by the Kolmogorov-Smirnov test, and logarithmic transformations were accordingly performed for nonparametric variables. The paired-sample *t*-test was used for assessing the effect of treatment in each group. ANCOVA, adjusted for baseline values, was used for comparisons between groups. All reported *P* values are based on two-sided tests with a significance level of 5%. Because of multiple comparisons we used Bonferroni's correction to account for the increase in type I error. Analyses were performed using the Statistical Package for the SPSS 15.0 (SPSS Inc, Chicago, IL).

## RESULTS

Recruitment took place from October 2009 to September 2011 and follow-up ended in December 2011. Initially, 100 Caucasian patients were enrolled ( $n = 33, 34$  and  $33$  in the switch to the highest dose of rosuvastatin, add-on ER-NA/LRPT and add-on fenofibrate group, respectively) (Figure 1). Ten patients dropped out due to side effects (see below). Eventually, ninety subjects (47 men,  $59 \pm 11$  years) completed the study ( $n = 32, 26, 32$  in the switch to highest-dose rosuvastatin monotherapy, add-on ER-NA/LRPT and add-on fenofibrate group, respectively) and included in the final analysis. No significant differences in baseline data were found across groups regarding demographic characteristics and serum

metabolic parameters (Table 1). Compliance rate was  $> 90\%$  in all participants who completed the study. No changes in body weight, dietary habits, antihypertensive or antidiabetic medications were reported during follow-up.

As previously reported, among study completers non-HDL-C decreased in all groups (by 23%, 24% and 7% in the rosuvastatin monotherapy, add-on ER-NA/LRPT and add-on fenofibrate group, respectively,  $P < 0.01$  for all *vs* baseline and  $P < 0.01$  for all *vs* fenofibrate group). LDL-C decreased by 23% and 19% in the rosuvastatin and ER-NA/LRPT group, respectively ( $P < 0.01$  *vs* baseline), but not in the add-on fenofibrate group.

As shown in Table 2, FPG increased in add-on ER-NA/LRPT and rosuvastatin monotherapy groups by 9.7% (from  $93 \pm 17$  to  $102 \pm 29$  mg/dL) and 4.4% (from  $91 \pm 26$  to  $95 \pm 12$  mg/dL), respectively ( $P < 0.01$  for all comparisons *vs* baseline and for the comparison between the 2 groups), while it did not significantly change in the add-on fenofibrate group (from  $98 \pm 11$  to  $98 \pm 12$  mg/dL,  $P > 0.05$  *vs* baseline). Of note one case of new onset DM was reported in the add-on ER-NA/LRPT group. HbA1c increased by 0.3% (from  $6.3\% \pm 0.7\%$  to  $6.6\% \pm 0.9\%$ ) in add-on ER-NA/LRPT group and by 0.2% (from  $6.1\% \pm 0.5\%$  to  $6.3\% \pm 0.5\%$ ) in the rosuvastatin monotherapy group ( $P < 0.01$  for all comparisons *vs* baseline and for the comparison between the 2 groups), while no significant change was reported in the add-on fenofibrate group (+ 0.1%, from  $6.3\% \pm 0.8\%$  to  $6.4\% \pm 1.0\%$ ,  $P > 0.05$  *vs* baseline). HOMA-IR increased by 65%

**Table 1** Baseline characteristics of patients who completed the study

	Switch to the highest dose of rosuvastatin	Add-on-statin ER-NA/LRPT	Add-on-statin micronised fenofibrate	P
Sex (males/females)	32 (17/15)	26 (14/12)	32 (16/16)	NS
Age (yr)	62 ± 10	58 ± 14	59 ± 12	NS
Hypertension	17 (53)	14 (54)	16 (50)	NS
Diabetes mellitus	6 (19)	6 (23)	6 (19)	NS
Metabolic syndrome <sup>1</sup>	17 (53)	15 (58)	18 (56)	NS
Smoking	10 (31)	9 (35)	9 (28)	NS
BMI (kg/m <sup>2</sup> )	29.1 ± 2.5	29.1 ± 3.1	28.8 ± 3.2	NS
TC				NS
(mg/dL)	205 ± 40	200 ± 42	200 ± 37	
(mmol/L)	5.5 ± 1	5.2 ± 1.1	5.2 ± 1	
Triglycerides				NS
(mg/dL)	190 (173-210)	213 (190-254)	218 (189-260)	
(mmol/L)	2.2 (2.0-2.4)	2.4 (2.2-2.9)	2.5 (2.1-2.9)	
HDL-C				NS
(mg/dL)	50 ± 10	47 ± 11	45 ± 9	
(mmol/L)	1.3 ± 0.3	1.2 ± 0.3	1.1 ± 0.2	
LDL-C				NS
(mg/dL)	116 ± 40	109 ± 35	112 ± 32	
(mmol/L)	3.2 ± 1.0	2.9 ± 0.9	2.9 ± 0.8	
Non-HDL-C				NS
(mg/dL)	155 ± 40	153 ± 37	155 ± 34	
(mmol/L)	4.0 ± 1.0	4.0 ± 1.0	4.0 ± 0.9	
Fasting plasma glucose				NS
(mg/dL)	91 ± 26	93 ± 17	94 ± 10	
(mmol/L)	5.1 ± 1.4	5.2 ± 0.9	5.2 ± 0.6	
HbA1c (%)	6.1 ± 0.5	6.3 ± 1.1	6.1 ± 0.8	NS
HOMA-IR index	1.4 1.2-2.1	1.5 1.4-2.1	1.7 1.5-2.3	NS
Medications at baseline				
Aspirin	9 (28)	8 (31)	7 (22)	NS
Beta blockers	9 (28)	8 (31)	9 (28)	NS
HCTZ	11 (34)	10 (38)	10 (31)	NS
ACEIs/ARBs	13 (41)	11 (42)	12 (38)	NS
Calcium channel blockers	8 (24)	8 (31)	10 (31)	NS
Metformin	6 (19)	6 (23)	5 (16)	NS
Pioglitazone	2 (7)	1 (4)	3 (9)	NS
Sulfonylurea	4 (13)	3 (12)	2 (6)	NS
Atorvastatin 5-20 mg/d	11 (34)	9 (35)	12 (38)	NS
Simvastatin 10-40 mg/d	11 (34)	9 (35)	9 (28)	NS
Rosuvastatin 5-10 mg/d	10 (31)	8 (31)	11 (34)	NS

Values are expressed as absolute mean ± SD [except for triglycerides and HOMA-IR index which are expressed as median (range)] or numbers (percentage).

<sup>1</sup>Participants who fulfilled 3 or more of the American Heart Association (AHA) criteria for the diagnosis of metabolic syndrome (waist circumference > 102 cm in men, > 88 cm in women, fasting serum triglycerides > 150 mg/dL, HDL-C < 40 mg/dL in men, < 50 mg/dL in women, blood pressure > 130/85 mm Hg, fasting serum glucose > 100 mg/dL). ACEIs: Angiotensin converting enzyme inhibitors; ApoB: Apolipoprotein B; ARBs: Angiotensin receptor blockers; BMI: Body mass index; ER-NA/LRPT: Extended release nicotinic acid/laropiprant; HCTZ: Hydrochlorothiazide; HDL-C: High-density lipoprotein cholesterol; HbA1c: Glycosylated hemoglobin; HOMA-IR index: Homeostasis model assessment of insulin resistance index; NS: Non significant; TC: Total cholesterol.

[from 1.5% (1.4%-2.1%) to 2.5% (1.5%-2.8%)] in add-on ER-NA/LRPT and by 14% [from 1.4% (1.2%-2.1%) to 1.6% (1.5%-2.6%)] in the rosuvastatin monotherapy group ( $P < 0.01$  vs baseline), while HOMA-IR level decreased in the add-on fenofibrate group [-6%, from 1.7% (1.5%-2.3%) to 1.6% (1.4%-2.2%),  $P < 0.01$  vs baseline and for the comparisons among the groups].

### Safety

Of the 100 patients enrolled, 8 (24%) of the 34 initially randomized to the ER-NA/laropiprant group dropped out during the study due to flushing ( $n = 5$ ), epigastric pain ( $n = 2$ ) and new onset diabetes ( $n = 1$ ). Also, 1 (3%) patient of the 33 initially randomized to the rosuvastatin group dropped out due to asymptomatic ALT elevation

> 3-fold ULN and 1 (3%) patient of the 33 randomized in the fenofibrate group due to serum creatinine elevation (> 20% from baseline) (Figure 1).

## DISCUSSION

We directly compared for the first time the switch to the highest dose of rosuvastatin versus add-on ER-NA/LRPT versus add-on micronised fenofibrate in patients with mixed dyslipidemia on a standard statin dose who had not achieved treatment goals. In the present prespecified analysis both add-on ER-NA/LRPT and switch to high-dose rosuvastatin were associated with glycemic profile deterioration, while add-on fenofibrate increased insulin sensitivity.

**Table 2** Laboratory parameters at baseline and 3 mo later

	Baseline	3 mo	Percentage change
Fasting plasma glucose, mg/dL (mmol/L)			
Switch to the highest dose rosuvastatin	91 ± 26 (5.1 ± 1.4)	95 ± 19 (5.3 ± 1.1)	4% <sup>b</sup>
Add-on-statin ER-NA/LRPT	93 ± 17 (5.2 ± 0.9)	102 ± 27 (5.7 ± 1.5)	10% <sup>b,d,f</sup>
Add-on-statin fenofibrate	94 ± 10 (5.2 ± 0.6)	94 ± 11 (5.2 ± 0.6)	0%
HbA1c, %			
Switch to the highest dose rosuvastatin	6.1 ± 0.5	6.3 ± 0.5	0.2% <sup>b</sup>
Add-on-statin ER-NA/LRPT	6.3 ± 1.1	6.6 ± 1.2	0.3% <sup>b,d,f</sup>
Add-on-statin fenofibrate	6.1 ± 0.8	6.2 ± 1.0	0.10%
HOMA-IR index			
Switch to the highest dose rosuvastatin	1.4 (1.2-2.1)	1.6 (1.5-2.6)	14% <sup>b,f</sup>
Add-on-statin ER-NA/LRPT	1.5 (1.4-2.1)	2.5 (1.5-2.8)	65% <sup>b,d,f</sup>
Add-on-statin fenofibrate	1.7 (1.5-2.3)	1.6 (1.4-2.2)	-6% <sup>b</sup>

Values are expressed as mean ± SD [except for fasting plasma insulin and HOMA-IR index which are expressed as median (range)]. No significant differences in baseline data were found across groups. <sup>b</sup>*P* < 0.01 *vs* baseline; <sup>d</sup>*P* < 0.01 *vs* switch to the highest dose rosuvastatin group; <sup>f</sup>*P* < 0.01 *vs* add-on-statin fenofibrate group. ER-NA/LRPT: Extended release nicotinic acid/laropiprant; HOMA-IR index: Homeostasis model assessment of insulin resistance index; HbA1c: Glycosylated haemoglobin.

Concerns regarding the effects of statins on glucose metabolism rose by the Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) study. An increase in the incidence of physician reported DM with rosuvastatin 20 mg/d in apparently healthy subjects with LDL-C < 3.36 mmol/L (130 mg/dL) and high-sensitivity C-reactive protein (hsCRP) > 2 mg/L was reported in this study<sup>[3]</sup>. A meta-analysis demonstrated a small increase in DM risk with statins with no evidence of heterogeneity across trials<sup>[4]</sup>. However, this estimate was attenuated and became no longer significant when the West of Scotland Coronary Prevention Study (WOSCOPS) was included<sup>[4]</sup>. A subsequent meta-analysis demonstrated that statin therapy was associated with a 9% increased risk for incident diabetes with little heterogeneity between trials<sup>[5]</sup>. Of note, this effect of statins seems to be dose dependent<sup>[4]</sup>. In fact, previously, we showed that rosuvastatin may increase insulin resistance and HOMA-IR levels in patients with impaired FPG in a dose-dependent manner and may increase the risk for new onset DM<sup>[6,7]</sup>. The mechanisms by which statins may adversely affect glucose homeostasis are not fully understood. Statins by inhibiting mevalonate pathway block the synthesis not only of cholesterol, but also of several mevalonate products known as isoprenoids<sup>[15]</sup>. Isoprenoids, including farnesyl pyrophosphate, geranylgeranyl pyrophosphate and ubiquinone, are known to enhance glucose uptake by upregulating the membrane transporter protein glucose transporter 4, which plays a key role in glucose uptake by adipocytes<sup>[15]</sup>. Therefore, statin-induced inhibition of isoprenoid synthesis may increase insulin resistance in the adipose tissue and induce hyperinsulinemia. Most potent statins in terms of inhibiting mevalonate pathway, such as rosuvastatin, as well as higher doses of statin treatment may exert a more profound impact on insulin resistance compared with conventional statins at low dosage regimens. Last, statins may exert a deleterious effect on insulin secretion by pancreatic islets<sup>[15]</sup>.

NA has been associated with modest, transient and

reversible elevation of FPG (an increase of approximately 4%-5%) and HbA1c levels (an increase of ≤ 0.3%)<sup>[16-18]</sup>. Although the mechanism remains unclear, an increase in insulin resistance seems to be involved. NA inhibits lipolysis in adipose tissue and decreases circulating free fatty acids (FFA). Paradoxically, the initially decreased FFA levels rebound during long-term NA treatment resulting in insulin resistance as elevated plasma FFA levels have been associated with insulin resistance<sup>[8]</sup>. Moreover, NA was associated with decreased expression of phosphoenolpyruvate carboxykinase (PEPCK1) in adipose tissue. PEPCK1 is a key enzyme in adipose tissue gluconeogenesis and its deficit leads to increased FFA release, partly explaining the rebound phenomenon<sup>[8]</sup>. Another contributing mechanism to FFA rebound may be the NA-induced up-regulation of tumor necrosis factor- $\alpha$  transcription and the consequent increase of interleukin-6, as both of them comprise cytokines with lipolytic properties. Apart from the FFA rebound, NA was also demonstrated to decrease protein kinase B and FOXO1 transcription factor phosphorylation. Both protein kinase B and FOXO1 are present in insulin-sensitive tissues and are involved in lipid and glucose metabolism. Of note, their phosphorylation is induced by insulin, while NA's opposite impact result in increased transcription of gluconeogenic enzymes and thus glucose overproduction and hepatic insulin resistance<sup>[8]</sup>. Of note, both body mass index and baseline FPG have been positively associated with the risk of new onset diabetes in non-diabetic patients receiving ER-NA<sup>[19]</sup>. In HPS-2 THRIVE (The Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events) trial patients (*n* = 25673) with established vascular disease who were already treated with simvastatin ( $\pm$  ezetimibe) were randomized to addition of ER-NA/LRPT (2000/40 mg/d) or placebo. After nearly 4 years of follow-up ER-NA/LRPT did not significantly reduce coronary deaths, nonfatal myocardial infarctions, strokes, or coronary revascularizations compared with statin ( $\pm$  ezetimibe) monotherapy<sup>[20]</sup>. What is more, diabetic complications

(typically hyperglycemia) were about twice as common as a reason for stopping randomized treatment in participants allocated to ER-NA/LRPT. Of note participants who developed DM were encouraged to continue their study treatment and this was rarely given as a reason for stopping<sup>[21]</sup>. As ER-NA/LRPT was associated with an excess of serious nonfatal side effects the drug was suspended worldwide<sup>[22]</sup>.

On the other hand, fenofibrate has been associated with increased insulin sensitivity and improved glycemic profile in patients with metabolic syndrome and impaired FPG, even though fenofibrate does not seem to affect insulin sensitivity in normolipidemic subjects<sup>[9,10,23]</sup>. Fenofibrate is a known peroxisome proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ) activator. Activated PPAR- $\alpha$  down-regulates lipid accumulation in liver and skeletal muscle decreasing hepatic very low density lipoprotein (VLDL) particles and subsequently levels of circulating FFA leading to increased insulin sensitivity<sup>[24]</sup>.

A major limitation of our study is its open-label design and the relatively small number of participants. On the other hand, it is an adequately powered randomized study with all laboratory determinations being performed blindly to treatment allocation. Also, study design is relevant to every day clinical practice.

Both addition of ER-NA/LRPT and switch to the highest dose of rosuvastatin deteriorated glycemic profile in patients with mixed dyslipidemia who were inadequately controlled with a standard statin dose. Add-on fenofibrate, on the other hand, improved insulin sensitivity. Larger prospective studies should address the effect of these treatment interventions on new onset diabetes incidence and cardiovascular disease risk.

## COMMENTS

### Background

Both cardiovascular disease and diabetes mellitus comprise major worldwide health problems with epidemic proportions and their prevention or delay is of major clinical significance. However, hypolipidemic treatment has been associated with deterioration of glycemic profile and even cases of new onset diabetes.

### Research frontiers

Lipid lowering treatments have been associated with controversial data regarding glucose metabolism. In this trial the authors demonstrated that both addition of extended release nicotinic acid/laropiprant and switch to the highest dose of rosuvastatin deteriorated glycemic profile in patients with mixed dyslipidemia who were inadequately controlled with a standard statin dose. Add-on fenofibrate, on the other hand, seems to increase insulin sensitivity.

### Innovations and breakthroughs

A number of studies and meta-analyses have suggested a potential diabetogenic role for both statins and nicotinic acid. The authors directly compared for the first time the effect on glycemic profile of the switch to the highest-dose of rosuvastatin with add-on nicotinic acid/laropiprant or add-on micronised fenofibrate in patients with mixed dyslipidemia on standard statin dose who had not achieved treatment goals.

### Applications

By knowing how lipid treatment may affect glucose metabolism in patients with mixed dyslipidemia inadequately controlled with a standard statin dose, the authors can choose on an individual patient basis the next therapeutic step taking under consideration both lipid and glycemic profile.

### Peer review

The authors examined the effect of hypolipidemic treatment on glycemic profile

in patients with mixed dyslipidemia. Both addition of extended release nicotinic acid/laropiprant and switch to the highest dose of rosuvastatin deteriorated glycemic profile, while add-on fenofibrate increased insulin sensitivity. Larger prospective studies should address the effect of these treatment interventions on new onset diabetes incidence and cardiovascular disease risk.

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## Influence of physiological and supraphysiological hyperinsulinemia on skin microcirculation in healthy volunteers

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### Abstract

**AIM:** To examine skin perfusion in dependency on insulinemia in healthy subjects.

**METHODS:** All volunteers were informed in detail about the procedures and signed informed consent. The protocol of this study was approved by the ethical committee. In our study, a two stage hyperinsulinemic euglycemic clamp was performed, with insulinemia 100 and 250 mIU/mL and glycemia 5.0 mmol/L (3% standard deviation). Before the clamp and in steady states, microcirculation was measured by laser Doppler flowmetry and transcutaneous oximetry and energy expenditure was measured by indirect calorimetry. Results (average and standard deviation) were evaluated with paired *t*-test.

**RESULTS:** Physiological (50 mIU/L) insulinemia led to higher perfusion in both tests; hyperemia after heating to 44%-1848% (984-2046) *vs* 1599% (801-1836), *P* < 0.05, half time of reaching peak perfusion after occlusion release 1.2 s (0.9-2.6) *vs* 4.9 s (1.8-11.4), *P* < 0.05. Supraphysiological (150 mIU/L) insulinemia led to even higher perfusion in both tests; hyperemia after heating to 44%-1937% (1177-2488) *vs* 1599% (801-1836), *P* < 0.005, half time to reach peak perfusion after occlusion release 1.0 s (0.7-1.1) *vs* 4.9 s (1.8-11.4), *P* < 0.005. A statistically significant increase occurred in tissue oxygenation in both insulinemia. The difference in perfusion and oxygenation between physiological and supraphysiological hyperinsulinemia was not statistically significant.

**CONCLUSION:** The post occlusive hyperemia test in accordance with heating test showed significantly increasing skin perfusion in the course of artificial hyperinsulinemia. This effect rises non-linearly with increasing insulinemia. Dependency on the dose was not statistically significant.

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**Key words:** Diabetes; Insulin; Laser Doppler flowmetry; Microcirculation

**Core tip:** Insulin mediated small vessel vasodilatation is a hot topic in microcirculation research. Our work describes skin microcirculation response to increasing insulinemia in the physiological and supraphysiological level. Simultaneous use of two laser Doppler flowmetry and transcutaneous oximetry was used for discriminating between total blood flow and perfusion of the nutritive bed. Both methods reveal increasing skin microcirculation perfusion due to insulin infusion with this effect

rising non-linearly with increasing insulinemia.

Krčma M, Čechurová D, Brožová J, Jankovec Z, Lacigová S, Žourek M, Rušavý Z. Influence of physiological and supraphysiological hyperinsulinemia on skin microcirculation in healthy volunteers. *World J Diabetes* 2013; 4(6): 372-377 Available from: URL: <http://www.wjgnet.com/1948-9358/full/v4/i6/372.htm> DOI: <http://dx.doi.org/10.4239/wjd.v4.i6.372>

## INTRODUCTION

Since the mid 1980s, a lot of attention has been dedicated to the importance of microcirculation, a part of the arterial bed including arterioles, precapillary sphincters, capillaries, venules and arteriovenous shunts. It is a structure of decisive importance for an organism; an exchange of blood gases and metabolic products takes place in its domain and it contributes to thermoregulation. Mediation of the vasomotor reaction and vasoarterial reflex maintaining a stable hydrostatic pressure is also an important function. Microcirculation is relatively difficult to access for more detailed examination due to its dimensions (capillary diameter approx.  $5 \times 10^{-5}$  mm<sup>2</sup>, blood flow velocity around 0.4 mm/s), yet its impairments are very severe and dominate in many metabolic disorders. Microcirculation impairment is crucial in diabetes mellitus, where arteriovenous shunts open at the expense of the nutritive bed due to a loss of sympathetic tone in peripheral circulation in diabetic neuropathy<sup>[1,2]</sup>. Blood flow is therefore seemingly sufficient, but the affected tissue undergoes ischemia (warm ischemia). To what extent hyperinsulinemia contributes to this effect is not yet clearly known; one of the possible explanations may be a stimulation of sympathetic activity. Several studies are dealing with insulin's vasodilatory effect with inconsistent findings regarding the extent of microcirculatory response at various insulin levels upon acute and chronic insulin administration.

Experimental work in rats showed an improvement in blood perfusion of sciatic nerve perineurium after a one month insulin treatment and concurrently, amelioration in nerve conduction was proven electromyographically<sup>[3]</sup>. A short-term continuous subcutaneous insulin infusion (insulin pump treatment) led to an increase in capillary perfusion<sup>[4]</sup> and to a decrease in venous oxygen tension in diabetic patients after 9 d of treatment. This result suggests an existence of redistribution of skin perfusion favoring the nutritive capillary bed.

Acute hemodynamic effects of insulin were tested in an experiment where healthy young men were treated with a short insulin, locally administered into brachial artery with a rate of 1 and 5 mU/min for a period of 90 min in a double blinded study design. Blood flow was measured using body plethysmography. A higher insulin dose led to a statistically significant vasodilatation (20%) compared to placebo. Administration of insulin + L-glucose (metabolically inactive stereoisomer) did not

produce a further increase in vasodilatation in comparison with pure insulin administration. Administration of insulin + D-glucose led to an increase in perfusion in comparison with pure insulin administration (47% compared to placebo). Glucose infusion itself did not cause any significant changes in blood flow<sup>[5]</sup>. In diabetes type 1 patients, when using different insulin infusion rates (1.5 IU/h, 15 IU/h), an increase in blood flow measured by laser Doppler flowmetry (LDF) at the low dose of insulin occurred, while a decrease occurred at the higher dose<sup>[6]</sup>. These measurements, however, were not performed in steady state conditions during clamp examination. Conversely, no statistically significant changes in perfusion of skin microcirculation were found in an experiment in healthy volunteers, where skin perfusion was monitored during a three step insulin clamp with gradually increasing insulinemia levels 60-500 IU/mL. Arteriovenous difference of glucose already attained a maximum value at the lowest insulinemia and subsequently remained constant<sup>[7]</sup>.

An increase in perfusion measured using LDF was noted at supraphysiological hyperinsulinemia in anesthetized rats under clamp conditions, concurrently associated with an increase in femoral artery flow<sup>[8]</sup>.

Some studies dealing with physiological hyperinsulinemia (approximately to 50 mIU/L) proved an increased blood flow through muscle and skin microvascular beds and increased density of opened capillaries under this condition<sup>[9,10]</sup>. They used LDF and video capillary microscopy for measurements. In addition, local skin administration of insulin using iontophoresis has a vasodilatory effect in healthy volunteers, which diminishes with age<sup>[11]</sup>, was not proven in diabetes type 2 patients and is decreased in obese non-diabetic women<sup>[12]</sup>. An improvement in glycemic control leads to an increase in microvascular reactivity in diabetes type 2 patients<sup>[13]</sup>.

To summarize the abovementioned findings, it is relatively well proven that physiological and even supraphysiological hyperinsulinemia leads to an increase in total limb blood perfusion. Study results regarding insulin influence on microcirculation vary. Some authors<sup>[7]</sup> suggest an insignificant role of physiological as well as supraphysiological insulinemia, others<sup>[6,9]</sup> observed an increase in perfusion only in physiological hyperinsulinemia. Furthermore, it is not completely clear what part of the vascular bed contributes to the increase in perfusion, whether it is an increase in blood flow through nutritive capillaries or arteriovenous shunts.

This study examined the vasodilatory effect of insulin on perfusion of skin microcirculation in healthy volunteers and assessed whether this effect follows a linear trend with insulinemia.

## MATERIALS AND METHODS

Microcirculation was examined at rest and after stimulation by physiologically (50 mIU/L) and supraphysiologically (150 mIU/L) increased level of insulin. Examinations was performed in 12 non-obese healthy volunteers

**Table 1 Characteristics of subjects**

Characteristics	Median (interquartile range)
Number (male/female)	12 (6/6)
Age (yr)	24 (23-25)
BMI (kg/m <sup>2</sup> )	21.6 (20.7-23.7)
Waist (cm)	74.5 (66.3-80.0)
Blood pressure (mmHg)	113/75 (107/66-117/80)
Fasting plasma glucose (mmol/L)	4.7 (4.6-5.3)
Plasma triglycerides (mmol/L)	0.8 (0.7-0.9)
HDL cholesterol (mmol/L)	1.4 (1.1-1.6)
LDL cholesterol (mmol/L)	2.5 (2.3-3.1)
Fibrinogen (mmol/L)	2.3 (2.2-2.5)

BMI: Body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

with no history of diabetes in parents and siblings, with no chronic disease or chronic medication except hormonal contraception in women, and matched in age as well as in basic anthropometric and biochemical parameters (Table 1). The study protocol was approved by the ethical committee of Medical Faculty in Pilsen, Charles University in Prague. All volunteers were fully acquainted in advance with the experiment and methods used, which they confirmed by signing an informed consent. The day before study commencement, the volunteers maintained an ordinary daily routine with the exception of heavy physical exercise, excessive consumption of carbohydrates, fats and alcohol, and last meal until 9 pm. The following morning, a two step hyperinsulinemic clamp with target insulinemia 50 and 150 mIU/L was performed according to a well-established method<sup>[14]</sup>, *i.e.*, rate of insulin infusion 2.4 and 6.0 IU/m<sup>2</sup> per hour. The order of insulinemias was inverted in one half of the subjects (*i.e.*, first 6.0 then 2.4 IU/m<sup>2</sup> per hour) and its sequence was random. We measured skin perfusion using LDF and transcutaneous oxymetry and respiratory quotient and energy expenditure by indirect calorimetry (V-max Sensormedics, Yorba Linda, CA, United States) according to a standard method<sup>[15]</sup> at basal conditions and in both steady states. M-value of each clamp was calculated to assess the change in insulin resistance. Results in the form of median and interquartile range were evaluated by the Wilcoxon test.

**Laser Doppler flowmetry**

Skin perfusion was examined at basal conditions before the clamp and in steady state at both insulin levels. System Periflux 5000 (Perimed, Sweden) with PF 5010 probe emitting laser with a wavelength of 780 nm and power output 1 mW was used for the measurement. The probe was placed to the dorsum of the non-dominant foot and measurement was performed in all subjects at a stable temperature of 33 °C. Subsequently, stimulation tests<sup>[16]</sup> were employed: heating (probe heating to 44 °C inducing maximal vasodilatation) and occlusion (3 min occlusion of a limb using a sphygmomanometer cuff inflated to a pressure of 30 mmHg higher than

**Table 2 Results are summarized in three groups**

	Basal	50 mIU/L	150 mIU/L
Measured insulin level (mIU/L)	3.5 (1.8-4.1)	47.5 <sup>b</sup> (36.0-53.3)	144.5 <sup>b</sup> (115.9-170.5)
LDF baseline (PU)	7.5 (6.8-10.2)	12.3 (9.2-21.8)	12.9 (8.6-29.9)
LDF heating test (%)	1599 (801-1836)	1848 <sup>a</sup> (984-2046)	1937 <sup>b</sup> (1177-2488)
LDF post-occlusion	4.9 (1.8-11.4)	1.2 <sup>a</sup> (0.9-2.6)	1.0 <sup>b</sup> (0.7-1.1)
Hyperemia test (s)	38.9 (35.5-40.8)	48.6 <sup>a</sup> (45.5-49.7)	57.4 <sup>a</sup> (51.7-66.2)
tcpO <sub>2</sub> (mmHg)			

Data are expressed as median (interquartile range). <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs basal group. LDF: Laser Doppler flowmetry.

systolic blood pressure), where time necessary for attaining maximal perfusion after cuff release was measured. These stimulation tests are a standard in examination of tissue perfusion<sup>[17-20]</sup> owing to a considerable time and spatial variability of plain basal perfusion measurement. Sampling rate was 31 ms and firmware Perisoft (Perimed, Sweden) was used for data evaluation.

**Transcutaneous oxygen monitoring**

Partial pressure of oxygen was measured using tcpO<sub>2</sub> probe PF 5040 of Periflux 5000 system (Perimed, Sweden), based on the principle of polarography<sup>[21]</sup>. A heated Clark electrode (45 °C) was attached to the skin of the foot dorsum at a standard location (between the 1<sup>st</sup> and 2<sup>nd</sup> metatarsus) using an adhesive ring and the space between the electrode and skin was filled with contact solution supplied by the producer. The probe was applied at least 10 min prior to measurement commencement. Sampling rate was 31 ms and firmware Perisoft (Perimed, Sweden) was used for data evaluation.

**RESULTS**

Data are clearly summarized in Table 2 and Figure 1. The group in which the clamp with lower target insulinemia was performed first did not statistically differ in baseline characteristics from the group with the initial higher insulinemic clamp. Statistically significant higher perfusion in skin microcirculation was achieved at physiological hyperinsulinemia in both tests [hyperemia after heating to 44 °C: 1848% (984-2046) vs 1599% (801-1836), P < 0.05; half time of reaching peak perfusion after occlusion release 1.2 s (0.9-2.6) vs 4.9 s (1.8-11.4), P < 0.05]. A statistically significant increase occurred in tissue oxygenation [tcpO<sub>2</sub>: 48.6 mmHg (45.5-49.7) vs 38.9 mmHg (35.5-40.8), P < 0.05].

The perfusion of skin microcirculation was even higher at supraphysiological hyperinsulinemia in both tests [hyperemia after heating to 44 °C: 1937% (1177-2488) vs 1599% (801-1836), P < 0.005, half time to reach peak perfusion after occlusion release 1.0 s (0.7-1.1) vs 4.9 s (1.8-11.4), P < 0.005]. A statistically significant increase

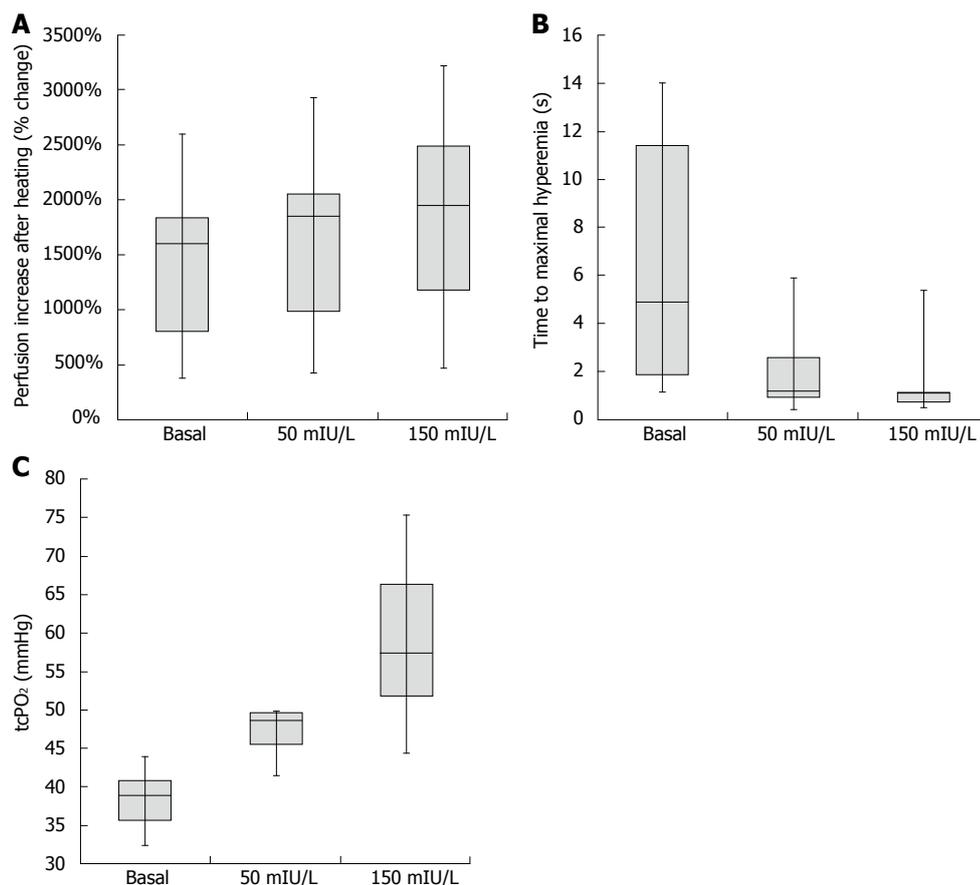


Figure 1 Heating (A), post-occlusive hyperemia (B) and transcutaneous oximetry (C) test results.

occurred in tissue oxygenation [tcpO<sub>2</sub>: 57.4 mmHg (51.7-66.2) *vs* 38.9 mmHg (35.5-40.8),  $P < 0.005$ ]. The difference in perfusion and oxygenation between physiological and supraphysiological hyperinsulinemia was not statistically significant. *M*-value measured during the clamp for insulin resistance evaluation did not change.

## DISCUSSION

Studies that monitored an influence of insulin on microcirculation used either local skin administration using iontophoresis<sup>[9,12]</sup> or systemic delivery<sup>[5-7]</sup>. The advantage of local administration is limited local hyperinsulinemia, which does not require a clamp examination associated with fluid infusion and change in hepatic production of glucose and pancreatic production of insulin. In our study, we chose the systemic administration with the advantage of physiological insulin distribution and elimination of influence of passage of electrical current, which can induce vasoconstriction *via* voltage-dependent sodium and calcium channels<sup>[22]</sup>.

Simultaneous use of LDF and transcutaneous oximetry was performed to distinguish perfusion of the nutritive bed (assessed through O<sub>2</sub> release) from total blood flow through microcirculation (including arteriovenous shunts) in the region of interest of the LDF probe, to which microvascular reactivity corresponds. However,

measuring transcutaneous partial oxygen pressure can only be considered a rough indicator of nutritive bed perfusion. The exchange of oxygen between the vascular bed and tissues also takes place on other levels (larger vessels *via* interstitial fluid) and a discrepancy was found between capillary density assessed through video capillary microscopy and transcutaneous oximetry values<sup>[23]</sup>.

In some older studies<sup>[6,7]</sup>, the authors describe no increase (or non significant increase) in microcirculation perfusion as a result of insulin infusion. On the other hand, more recent studies<sup>[9,10]</sup> demonstrate an increase in perfusion at physiological hyperinsulinemia. The explanation may be due to different methodology being used. In older studies, perfusion was measured by a probe only at basal conditions and no stimulation test was employed. According to our findings, the value of basal LDF perfusion showed only an insignificant incremental trend (Table 2), which corresponds to data measured earlier.

Transcutaneous oxygen pressure monitoring is important for estimation of amputation wound healing in diabetic foot syndrome<sup>[24]</sup> as well as for angioplasty effect monitoring in patients with critical limb ischemia<sup>[25]</sup> with tcpO<sub>2</sub> values at rest below 30 mmHg as an independent predictor of ischemia<sup>[26]</sup>. The increase in transcutaneous oxygen pressure observed in our study is consistent with previous study data<sup>[4]</sup>, where arteriovenous difference

of oxygen increased with continuous subcutaneous insulin infusion, suggesting a flow redistribution favoring a functional vascular bed. On the contrary, in patients with diabetes mellitus type 2 with insulin resistance and hyperinsulinemia present, transcutaneous oxygen pressure is inversely proportional to insulinemia and it falls with its increase<sup>[27]</sup>. In obese patients with metabolic syndrome but without diabetes, decreased vasomotion and reduced response to locally administered insulin was described<sup>[10]</sup>. These findings suggest a different behavior of microcirculatory vascular bed in hyperinsulinemic insulin-resistant patients, where the response of microcirculation to exogenous insulin administration is altered and there is no improvement of nutritive perfusion (possibly even deterioration), while in insulin-sensitive patients, insulin administration causes more nutritive capillaries to open. Pathogenesis mechanism of reactivity changes is put in connection with oxidative stress induced by hyperlipidemia and insulin resistance, which causes vasoconstriction through augmentation of endothelin receptor activity (for thromboxane A2) in smooth muscle tissue<sup>[28]</sup>. In this regard, it will be interesting to observe insulin mediated microcirculation redistribution in patients with chronic heart failure treated with newly developed endothelin receptor antagonists; such a study has not yet been done according to the available literary data. There was no true control group with only fluid intake (without glucose and insulin) in our study. This can be considered a limitation of the study because the most important question is whether the fluid load associated with clamp examination itself does not lead to sympathetic activation and microcirculation reactivity increase.

In the literature, we can find a mention about a slight microcirculatory reactivity increase associated with fast infusion of saline<sup>[13]</sup>. In our experiment, healthy volunteers always had a total of 1-1.5l of glucose solution administered during the 2 h clamp and no increase in heart rate was observed. This rate of fluid infusion cannot be considered sufficient to trigger a sympathetic response. In addition, no significant difference in measured parameters was observed between the two subgroups (initially lower and higher insulinemia) despite the fact that the administered fluid volume at the first and the second steady state varied. Therefore, we presume the potential effect of fluid intake as insignificant. Hyperinsulinemia causes an increase in reactivity of microcirculation as well as an increase in transcutaneous oxygen pressure in healthy volunteers upon systemic administration of insulin. This effect rises non-linearly with increasing insulinemia.

## COMMENTS

### Background

Insulin, as a most potent antidiabetic drug, is currently studied from many points of view. One of them is the influence on microcirculation and whether (and how much) insulin blood concentration contributes to vasodilatation.

### Research frontiers

There are new technologies for measuring microvasculature perfusion, laser

Doppler flowmetry and transcutaneous oximetry. Both of them contribute to distinguishing between nutrition and thermoregulation vessels.

### Innovations and breakthroughs

This work describes the microcirculation response to increasing insulinemia; perfusion is increased in both physiological and supraphysiological levels.

### Applications

In contrast to large vessel examination, microcirculation examination is quite complicated. The mentioned techniques can help to measure drug mediated microcirculation perfusion in acute and chronic states.

### Peer review

The authors investigated the vasodilatory potential of insulin on skin microcirculation in healthy volunteers. This is an interesting study but the authors need to include a description of statistical methods and specific statistical tests.

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