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EDITORIAL

Saliva as a non-invasive diagnostic tool for inflammation and insulin-resistance

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Abstract

Saliva has been progressively studied as a non-invasive and relatively stress-free diagnostic alternative to blood. Currently, saliva testing is used for clinical assessment of hormonal perturbations, detection of HIV antibodies, DNA analysis, alcohol screening, and drug testing. Recently, there has been increasing interest in evaluating the diagnostic potential of saliva in obesity, inflammation, and insulin-resistance. Current literature has demonstrated elevated levels of inflammatory biomarkers including C-reactive protein, tumor necrosis factor-α, interleukin-6, and interferon-γ in saliva of obese/overweight children and adults. Salivary antioxidant status has also been studied as a measure of oxidative stress in individuals with type 2 diabetes. Further, several studies have demonstrated correlations of salivary markers of stress and insulin resistance including cortisol, insulin, adiponectin, and resistin with serum concentrations. These findings suggest the potential diagnostic value of saliva in health screening and risk stratification studies, particularly in the pediatric population, with implications for inflammatory, metabolic and cardiovascular conditions. However, additional studies are required to standardize saliva collection and storage procedures, validate analytical techniques for biomarker detection, and establish reference ranges for routine clinical use. The purpose of this review is to summarize and evaluate recent advancements in using saliva as a diagnostic tool for inflammation and insulin-resistance.

Key words: Saliva; Inflammation; Cytokines; Insulin resistance; Adipokines

Core tip: Recent studies have shown that salivary concentrations of several inflammatory cytokines and insulin resistance indices (which may be lower than serum concentrations) may mirror alterations in systemic concentrations of such biomarkers. Saliva offers a promising diagnostic alternative, compared to blood sampling, for screening for inflammatory, metabolic, and cardiovascular risk factors particularly among pediatric and geriatric populations where blood sampling may be difficult. Additional research is needed to validate salivary biomarkers and establish reference ranges and characterize the influence of diet, physical activity, and drug treatment.

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SALIVA AS A DIAGNOSTIC TOOL: CURRENT KNOWLEDGE

Saliva, an exocrine secretion of the salivary glands, containing water (99%), electrolytes, proteins, and enzymes,

provides sensory perception of food, and aids chewing, swallowing, and digestion of food[1]. Saliva protects tissues against desiccation, penetration, ulceration, potential carcinogens, and assists in wound healing[2]. Whole saliva comprises of a mixture of fluids, secreted from the salivary glands (submandibular, sublingual, and parotid, and the minor gland), gingival fold, oral mucosa transudate, and, mucous from the nasal cavity and pharynx, that vary in rheological properties and the composition of their secretions[3-6]. The parotid gland secretions are largely composed of water and electrolytes, while the submandibular and sublingual glands produce both serous and mucous secretions, with mucin being the most abundant protein in saliva[7]. Saliva also contains cystatins, proline-rich peptides, and other molecules that are found in blood[4,8]. Saliva is hypotonic to plasma and is actively involved in exchange of sodium (Na^+), chloride (Cl^-), potassium (K^+) and bicarbonate ($HCO3^-$) ions with plasma[7]. Proteins and other substances from blood have been shown to enter saliva intracellularly through passive diffusion or active transport, and paracellularly through ultrafiltration at tight junctions between cells[9]. Saliva can be collected by passive drool technique or by using oral swabs. In healthy individuals, depending on age and gender, the unstimulated salivary flow rate is between 0.1-2 mL/min[10]. Additional factors influencing unstimulated salivary flow and composition include individual hydration, body posture, lighting, smoking, circadian and circannual rhythms, and medications[1].

The use of saliva as an alternative diagnostic tool to blood offers certain advantages. Salivary composition has been observed to be influenced by systemic changes allowing identification of biomarkers for disease conditions. Since saliva collection is non-invasive and relatively stress-free, saliva can serve as a potential alternative diagnostic fluid in infants, toddlers, youth and adults. However, despite its diagnostic potential, saliva has not yet been established as an analytical tool due to insufficient information regarding salivary biochemical composition and its correlation with plasma levels. Salivary Na, K, total protein, IgA and amylase activity has been shown to increase linearly with age. For example, salivary amylase activity has been shown to be variable and significantly different between infants and toddlers[11]. However, in healthy adults (mean age 22 years), no significant differences were observed in salivary concentrations of glucose, inorganic phosphate, total protein, Mg^{2+}, Cl^- and Ca^{2+} between men and women participants[12]. Interestingly, recent studies demonstrate the diagnostic utility of saliva with implications for cardiovascular disease, systemic and local inflammation, hepatic damage and insulin resistance[8,13,14].

Currently, saliva testing is used in areas of toxicology, endocrinology, infectious diseases, and forensics, with established diagnostic tests available for alcohol detection, HIV infections, hormonal analyses, and drug testing[15,16]. Several studies have demonstrated the use of saliva for detection of antibodies against HIV-1 and HIV-2 under non-laboratory settings[17,18]. The United States Food and Drug Administration (FDA) has recently approved OraQuick, the first over-the-counter, in-home self-testing HIV kit, which uses an oral sample for rapid detection of antibodies against HIV[19]. The assessment of hormones in saliva has been widely studied for routine clinical use[20-22]. The FDA has recently approved the use of enzyme immunoassay technique for *in vitro* diagnostic assay of salivary cortisol for adrenal cortical function and screening for Cushing's and Addison's disease[23]. In this review, we explore the potential of using saliva as a non-invasive diagnostic tool for the measurement of biomarkers of insulin-resistance and inflammation.

GLUCOSE IN SALIVA

Salivary glucose has been shown to significantly correlate ($r = 0.5216$, $P < 0.05$) with serum glucose in healthy subjects ($n = 15$). In individuals with newly diagnosed type 2 diabetes ($n = 106$), salivary glucose demonstrated strong correlation with serum glucose ($r = 0.7686$, $P < 0.01$) and serum HbA1c ($r = 0.5662$, $P < 0.01$). Type 2 diabetic patients had significantly higher ($P < 0.01$) mean salivary glucose values (4.22 ± 3.59 mg/mL) compared to healthy controls (1.23 ± 0.52 mg/mL)[24]. Pendyala *et al*[25] have also evaluated serum and salivary glucose in diabetic (men = 26, women = 14) and non-diabetic (men = 28, women = 12) individuals[25]. These authors observed significant correlation between fasting salivary and plasma glucose in both diabetic ($r = 0.40$) and non-diabetic ($r = 0.58$) groups. Further, they reported a significant difference in fasting salivary glucose ($P < 0.001$) between diabetic (10.93 ± 1.93 mg/mL) and non-diabetic controls (6.08 ± 1.16 mg/mL). Further, a recent systematic review reported a meaningful increase in salivary glucose concentration in type 2 diabetes that was associated with HbA1c values, suggesting that salivary glucose levels may be a potential biomarker for type 2 diabetes mellitus[26]. Ongoing research is focused on the development of nanotechnology-based biochip sensors for salivary glucose measurements. Such a novel biochemical sensor that provides a compact, high-throughput device for real-time glucose measurements may have implications in point-of-care clinical settings[27].

INSULIN IN SALIVA

Salivary insulin, assayed in normal and type 1 diabetic subjects by Pasic and Pickup demonstrated significant correlation between mean serum insulin and salivary insulin ($r = 0.81$, $P < 0.01$ in non-diabetics and $r = 0.91$, $P < 0.001$ in type 1 diabetics)[28]. However, because several individual profiles showed marked discrepancies between the timing and magnitude of insulin changes, these authors did not recommend salivary insulin concentrations as a reliable index of insulinemia. More recently, studies by Fabre *et al*[29] demonstrated that salivary insulin concentrations were approximately 10 times lower than

serum insulin concentrations[29]. These authors showed a significant correlation (r = 0.92, P < 0.001) between salivary and serum insulin concentrations in 130 boys and 147 girls, aged 6-14 years, suggesting that salivary insulin measurements may be a feasible approach, but suggest the need for additional studies to validate these findings. However, there were no reports that assessed surrogate measures of insulin resistance, including the Homeostasis Assessment Model-estimated insulin resistance (HOMA-IR) or the Quantitative Insulin Sensitivity Check Index[30,31].

CORTISOL IN SALIVA

One of the most widely studied salivary biomarker of stress is the glucocorticoid hormone, cortisol[32,33]. Elevated cortisol production can lead to hypertension, central obesity, insulin resistance and glucose intolerance[34]. In a study of overweight Latino youth (n = 211, boys = 119, girls = 92, age between 8 and 13 years) at risk for type 2 diabetes, cortisol was shown to negatively influence insulin sensitivity, and was inversely correlated with fasting glucose (r = 0.23, P < 0.01), β-cell function (r = -0.24, P < 0.05), and acute insulin response to glucose (r = -0.27, P < 0.05)[35]. HPA-axis dysfunction has been associated with various psychological and pathophysiological conditions, and hyperactivity of hypothalamic-pituitary-adrenal (HPA) axis has been observed in individuals with type 2 diabetes[36,37].

Saliva contains free, biologically active cortisol as opposed to total cortisol present in serum or plasma. Further, the concentration of cortisol in saliva is independent of the salivary flow rate and is strongly correlated with circulating cortisol concentrations[33,36]. Cortisol follows a diurnal pattern and any disruption in the rhythm would also be indicative of an HPA dysfunction. The average salivary cortisol concentrations in healthy subjects were reported to be higher in the morning (0.20-1.41 μg/mL) compared to afternoon values (0.04-0.41 μg/mL)[33]. Björntorp *et al*[36] have reported the use of salivary cortisol measurements to monitor the activity of HPA axis. In their study, circulatory perturbations in cortisol expression, which are indicative of increased risk of endocrine abnormalities, insulin resistance, central obesity, dyslipidemia, hypertension and type 2 diabetes, were reflected in the salivary cortisol levels[36].

Data from the Multi-Ethnic Study of Atherosclerosis has demonstrated associations between salivary cortisol and markers of inflammation including interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF)-α in plasma[38]. In this study, IL-6 was found to be most consistently related to cortisol profiles, and higher IL-6 levels were inversely associated with lower cortisol awakening response. In obese individuals (men, n = 91; women, n = 103) between the ages 19 to 35 years, significant associations were observed between cortisol levels and body fat distribution[39].

Salivary cortisol concentrations are known to increase within 5 min of increases in plasma cortisol, and are generally well correlated with plasma values[40]. There are several salivary cortisol kits available commercially, which commonly use immunoassay techniques or the more recent liquid chromatography-tandem mass spectrophotometry technique. In clinical settings, salivary cortisol is frequently used in the diagnosis of Cushing's syndrome with reported sensitivities and specificities of 90%[41,42]. Saiyudthong *et al*[43] have conducted a study to compare salivary cortisol levels in healthy individuals (n = 83, aged 18-25 years), measured by enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescence (ECL). Salivary cortisol showed a positive correlation with serum values (r = 0.84, P < 0.001) measured using ECL. Further, there was no significant difference between salivary cortisol measured by ELISA and ECL, suggesting ECL as an alternative detection technique for salivary cortisol measurement[43].

ADIPOKINES IN SALIVA

Adipose tissue produces several pro-inflammatory and anti-inflammatory factors, including the adipokines leptin, adiponectin, resistin, and visfatin, as well as cytokines such as TNF-α, IL-6, and chemokines such as monocyte chemoattractant protein-1 (MCP-1). These have been shown to participate in the pathogenesis of insulin resistance, adipogenesis and inflammation[44-48].

Recent studies have shown that resistin, visfatin, and adiponectin concentrations can be measured using saliva (Figure 1)[45,46,49]. Mamali *et al*[45] have examined associations between serum and salivary concentrations of adiponectin, resistin and visfatin in healthy individuals (men, n = 17; women, n = 33) with a mean age of 34 ± 14 years, body mass index (BMI) 22.4 ± 3.6 and body fat percentage 22.4 ± 8.4. In this study, mean salivary (10.92 ng/mL) and serum (12.27 μg/mL) adiponectin levels were shown to be marginally correlated (r = 0.347, P = 0.019). There was a significant positive correlation (r = 0.441, P < 0.01) between salivary (1.69 ng/mL) and serum (7.78 ng/mL) resistin values, and no statistical correlation between salivary (9.51 ng/mL) and serum (21.41 ng/mL) visfatin values[45]. Further, the study reported that the differences were not significant between men and women. Similarly, Toda *et al*[50] have demonstrated significant correlation (P < 0.05) between plasma and salivary adiponectin values in healthy female participants (n = 30, age > 43 years)[50]. In this study, the authors have compared plasma adiponectin (11.7 μg/mL) concentrations with salivary adiponection in saliva samples collected directly in a test tube (0.89 ng/mL), and with cotton wads using the Salivette system (0.82 ng/mL). There was a significant correlation (P < 0.05) between plasma and test-tube saliva samples, and not with the Salivette samples. Salivary detection of proteins such as adiponectin depends largely on salivary processing methods, and the recovery of proteins from saliva. Thanakun *et al*[51] have demonstrated filtration as an alternative saliva processing technique, to the commonly used centrifugation method. In this study,

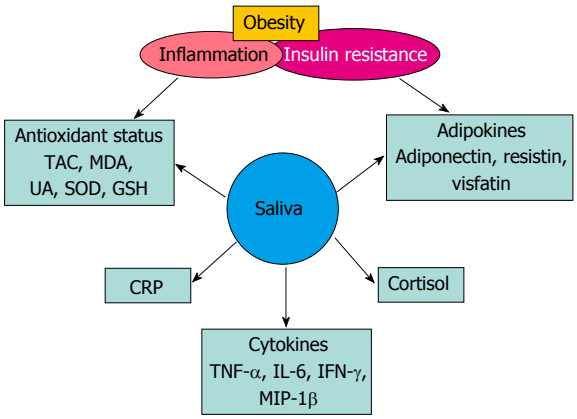

Figure 1 Salivary biomarkers of inflammation and insulin resistance. TAC: Total antioxidant capacity; MDA: Malondialdehyde; UA: Uric acid; SOD: Superoxide dismutase; GSH: Glutathione reductase; CRP: C-reactive protein; TNF-α: Tumor necrosis factor-alpha; IL-6: Interleukin-6; IFN-γ: Interferon gamma; MIP-1β: Macrophage inflammatory protein-1 beta.

adiponectin levels, following filtration, were comparable to those after centrifugation[51]. In another study, these authors have demonstrated significant association ($r = 0.211$, $P = 0.018$) between salivary and plasma adiponection, using ELISA technique, in both healthy individuals ($n = 46$) and patients with metabolic syndrome ($n = 82$). The authors, however, did not observe significant difference in salivary adiponectin between the 2 study groups[52].

In a second study, Yin *et al*[46] have reported significantly higher salivary resistin concentrations ($P > 0.05$) in individuals with newly diagnosed type 2 diabetes (men, $n = 18$; women, $n = 20$) compared to non-diabetic subjects. Salivary resistin was significantly correlated with serum resistin concentrations at different time points of oral glucose tolerance test, and was not affected by an oral glucose load. Further, there was a positive correlation of serum and salivary resistin concentrations with BMI and HOMA-IR in both control and diabetic groups[46]. The studies together indicate that while assay validation and the method of saliva sample collection can play a key role in biomarker quantification and standardization, saliva has the potential to be further explored as a diagnostic tool for adipokine analyses. More research needs to be directed towards developing saliva processing techniques, which can substantially increase the recovery of proteins. Higher protein yields can positively contribute towards improving outcomes of studies determining correlations between saliva and serum concentrations of adipokines.

INFLAMMATORY BIOMARKERS IN SALIVA

Inflammation can be caused by a variety of conditions including oxidative stress, overweight/obesity, improper oral hygiene and nutritional deficiencies[1,13,53]. Chronic low-grade inflammation has been associated with systemic diseases, insulin resistance and development of type 2 diabetes[54,55]. Focusing on the need to establish rapid, non-invasive and easy-to-use strategies for disease diagnosis, there has been growing interest in evaluating the potential of saliva for inflammatory marker profiling.

Studies have indicated that the most commonly explored biomarkers of inflammation include antioxidant status and C-reactive protein (CRP) concentrations[53,56-58]. Spectrophotometric assays quantifying levels of thiobarbituric acid reacting substances (TBARS) are used to evaluate salivary antioxidant status, while CRP concentrations are measured using ELISA kits or high-sensitivity immunoturbidimetric assays[53,56-59]. However, these tests lack the sensitivity for detection of CRP in saliva. To address the issue of sensitivity, researchers have developed a "lab-on-the-chip" technique for salivary CRP measurements. This novel technique utilizes a microchip assay system that offers the advantages of increased sensitivity (10 pg/mL of CRP) with lower noise-to-signal ratio. The lab-on-the-chip system captures optical signals generated by chemical and immunological reactions performed on microspheres (280 microns in diameter) implanted in silicon microchip wells[60]. Saliva collection techniques reported in clinical studies include the use of unstimulated passive drool or the filter paper method[59,61]. However, it has been observed that correlations between salivary biomarkers were not strong enough to support one collection method over another[61].

Williamson *et al*[61] have reported the presence of 27 cytokine biomarkers including IL-1β, IL-1 receptor agonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, eotaxin, basic fibroblast growth hormone, growth-colony stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interferon-inducible protein 10, MCP-1, macrophage inflammatory proteins (MIPs)-1α, MIP-1β, platelet-derived growth factors BB, TNF-α, and vascular endothelial growth factor in the saliva of healthy adults. These cytokines were measured using a commercially available cytokine multiplex assay kit that combines the use of fluorescent flow cytometry and ELISA technology. These authors observed that out of the 27 cytokines tested, only 3 cytokines including IL-6, IFN-γ and MIP-1β, found in saliva samples collected by passive drool, showed significant correlation ($P < 0.05$) with plasma levels[61].

Recently, a novel clinical approach termed, salivary transcriptome diagnostics, has been evaluated to provide a robust, high-throughput and reproducible tool for salivary biomarker detection. Using microarray analysis and quantitative polymerase chain reaction, this method has demonstrated high sensitivity (91%) and specificity (91%) for inflammatory biomarkers including IL-8 and IL-1β[62]. Another emerging technique called the oral fluid nanosensor test (OFNASET), offers a rapid and simultaneous detection of multiple salivary proteins, including IL-8 and IL-1β, for point-of-care disease screening and detection. OFNASET involves the use of advanced electrochemical-based molecular analysis platforms including self-assembled monolayers, bionanotechnology, cyclic

enzymatic amplification, microfluids, hybridization-based detection, and molecular purification[63].

A recent study in healthy adolescent girls (11-17 years), observed that cytokines including GM-CSF, IL-1β, IL-2, IL-6, IL-8, IL-12p70, TNF-α, adiponectin, and cotinine were detectable in saliva. However, the cytokine concentrations, except IL-8 and IL-1β, were lower than serum values and variable at baseline. Further, there were no serum-saliva associations in the levels of cytokines tested[64]. It has been suggested that lack of correlation between salivary and plasma cytokine biomarkers may be due to the impact of oral environment, and the influence of local immunity. It has also been indicated that the variability in cytokine levels may be due to distinct diurnal patterns, reflecting the time of saliva collection[65].

Salivary concentrations of TNF-α and IL-6 have been shown to be elevated in individuals with type 2 diabetes and periodontal disease (n = 20, mean age = 57 ± 4 years), compared to healthy subjects (n = 21) with periodontal disease[66]. In this study, salivary TNF-α and IL-6 were assayed with ELISA-sandwich technique using commercially available immunoassay kits. In type 2 diabetic patients with periodontal disease, both salivary and serum TNF-α and IL-6 concentrations were significantly higher compared to healthy individuals with periodontal disease. Further, there was a significant correlation (r = 0.500, P = 0.057) between salivary and serum IL-6 concentrations, and between salivary IL-6 and parameters including age, BMI, blood glucose and HbA1c. Salivary TNF-α also showed a significant positive correlation (r = 0.674, P = 0.0006) with serum concentrations in diabetics with periodontal disease. However, salivary TNF-α was not correlated with age, BMI, blood glucose and HbA1c.

In overweight and obese children (mean age 14.5 years), BMI adjusted for age and gender was shown to be significantly associated with reduced flow rate of stimulated whole saliva (1.2 mL/min), compared to the salivary flow rate (2.0 mL/min) in normal-weight children. This suggested that childhood obesity may cause stimulated whole saliva flow rate to fall below the median value of 1.5 mL/min, which can negatively impact oral health in children[67]. Further, overweight and obese children, between 7 and 10 years of age, have demonstrated a significant decrease in salivary concentrations of phosphate (P < 0.001) and peroxidase activity (P < 0.001), and an increase in free sialic acid (P = 0.004) and protein (P = 0.003) levels compared to normal weight control group suggesting the influence of BMI on stimulated whole saliva composition[68].

CRP IN SALIVA

CRP is a sensitive marker of systemic inflammation and an independent risk factor for cardiovascular diseases in both adults and children[69,70]. In a study of 170 black South African children (age 10 ± 2 years; boys, n = 70; girls, n = 100) salivary CRP concentrations, determined using a commercially available CRP ELISA kit, showed that obese children (n = 53, boys = 24, girls = 29, mean BMI = 26.2 ± 5 kg/m^2) had significantly higher (P < 0.05) salivary CRP concentration (7.31 ± 0.93 pg/mL) compared to normal-weight control group (6.77 ± 0.92 pg/mL)[57]. Further, obese children were also shown to have significantly higher (P < 0.05) salivary CRP secretion rate (7.25 ± 0.99 pg/min) compared to normal weight children (6.68 ± 0.98 pg/min).

In healthy individuals (men, n = 13; women, n = 12) between 20 to 35 years age, salivary CRP concentrations have been shown to be in the range of 35-217 pg/mL for saliva collected using the passive drool method. Use of acid-stimulation for saliva collection have shown lower salivary CRP concentrations (38-171 pg/mL) compared to saliva collected using mechanical stimulation (32-213 pg/mL)[71]. In this study, a commercially available ELISA kit (AlphaLISA, PerkinElmer, MA, United States) was used for quantification of salivary CRP. Another study has reported salivary CRP concentrations in the range of 118 to 24156 pg/mL in healthy participants (n = 61) between 20 and 54 years of age[72]. In this study, saliva samples were collected using the unstimulated passive drool method and salivary CRP was measured with a commercial ELISA kit (Salimetrics LLC, Carlsbad, CA). The observed differences in salivary CRP range among healthy individuals may be explained by differences in pre-processing techniques, and the use of different assay kits. Further, these authors have shown that salivary and serum CRP concentrations were correlated (r = 0.72). Further, it was shown that salivary CRP concentrations could predict serum CRP concentrations with 89% accuracy at higher mean serum values.

However, Qvarnstrom *et al*[58] have reported that salivary CRP was not significantly associated with metabolic syndrome in patients with or without coronary artery disease[58]. In this study, out of 250 participants with coronary artery disease, 81 had metabolic syndrome, and salivary lysozyme was shown to be significantly associated with metabolic syndrome (P = 0.02), independent of CRP concentrations. While comparing saliva and plasma CRP concentrations, Dillon *et al*[59] have reported that CRP concentrations in saliva of healthy adults (n = 69) ranged between 0.05 to 64.3 μg/L, which were significantly lower compared to plasma CRP concentrations (0.14 to 31.1 mg/L). Further, regression analysis showed no correlation between CRP concentrations in saliva and plasma (R^2 = 0.001)[59]. In this study, unstimulated whole saliva samples were obtained by the passive drool method and salivary CRP concentrations were measured using a commercial kit (Salimetrics). Interestingly, salivary CRP concentrations have been shown to be positively correlated with serum concentrations in patients (n = 56) with acute myocardial infarction[73]. In this study, CRP showed the highest median concentration, for diseased over control subjects, in both serum (4.29) and saliva (72.25) followed by matrix metalloproteinase-9, IL-1β, soluble intercellular adhesion molecule 1, myeloperoxidase, adiponectin and MCP-1. Receiver-operating characteristic curve analysis showed that CRP had a significantly higher

area under the curve for saliva (area under the curve = 0.78, $P < 0.05$). The current developments in identifying and standardizing potential inflammatory biomarkers in saliva suggest that substantial research is required to standardize and validate the use of clinically relevant biomarkers in disease diagnosis[74].

ANTIOXIDANT STATUS IN SALIVA

Oxidative stress is another major cause of obesity-induced inflammation resulting from increased production of free radicals and/or low antioxidant status. Oral inflammation is associated with elevated systemic inflammation, and has been linked with increased risk of insulin resistance and diabetes[24]. In a study by Al-Rawi[56], the oxidative status of type 2 diabetic patients was evaluated by measuring salivary and serum levels of malondialdehyde (MDA), uric acid (UA), superoxide dismutase and reduced glutathione (GSH). Salivary concentrations of MDA were lower (between 0.29-0.98 μmol/L) compared to serum MDA values (0.85-4.31 μmol/L) in all the study groups. However, salivary MDA was significantly higher in participants with type 2 diabetes compared to control subjects. Further, UA and GSH concentrations were significantly elevated ($P < 0.001$) in saliva of diabetic patients, while salivary GSH showed no significant change compared to the control group[56].

Type 2 diabetes has also been associated with decreased total antioxidant capacity (TAC) evaluated by spectrophotometric measurement of TBARS[25]. In this study, salivary TAC content (1.24 ± 0.18) was significantly lower in diabetes group ($n = 30$, 13 men and 17 women) compared to healthy controls ($n = 30$, 4.6 ± 0.31). Further, there was a significant decrease ($P < 0.01$) in the salivary flow rates in subjects with diabetes (0.38 ± 0.16) compared to the healthy individuals (0.65 ± 0.10). A recent study has demonstrated increased concentrations of pro-inflammatory cytokines in unstimulated whole saliva samples collected from pregnant women with diabetes ($n = 63$). The findings of this study suggested that changes in saliva properties were more pronounced in long-term cases of diabetes and partly correlated with HbA1c[75].

SALIVA RESEARCH: EMERGING STUDIES

Currently, there has been an increasing focus on proteomic analysis of saliva. Research is directed to identify and catalog human salivary proteins. Recently, a NIDCR-supported research consortium has compiled an extensive list of whole saliva proteins, using mass spectrophotometric techniques. This research group has identified 597 salivary proteins that are also found in the plasma[76].

Studies are being conducted to develop sensitive and reliable saliva-based diagnostic assays with the potential to be used in a clinical setting. Researchers have evaluated the use of a Luciferase Immunoprecipitation System for detection of autoantibodies in salivary and lacrimal gland secretions of patients with Sjogren's Syndrome (SjS)[27]. This assay has been reported to detect autoantibodies in 67% of SjS patients with 100% specificity suggesting its potential use as an alternative to serum.

Interestingly, approximately 50 microRNAs have been identified in whole saliva, which currently are being studied for their potential to serve as biomarkers of oral cancer[77]. Scientists have also developed a surface immobilized optical protein sensor to detect IL-8 with implications for use in cancer detection. To overcome the challenge of detecting low concentrations of biomarkers in saliva, the authors propose use of confocal optical sensors[78].

CONCLUSION

While low-grade inflammation, a hallmark of obesity, may be a pivotal mechanism linking obesity to its numerous systemic complications, these require invasive procedures, such as blood drawing. Recently, interest in the use of saliva as a diagnostic fluid has increased exponentially because of its non-invasive nature and potential to be used in population-based screening programs, confirmatory diagnosis, risk stratification, prognosis determination, and therapy response. Salivary cortisol is becoming widely used as a screening test for the diagnosis of hypercortisolism and as a biomarker of psychological stress. Current literature for diagnostic potential of salivary biomarkers suggests that salivary CRP, TNF-α, IL-6, and IFN-γ are elevated in overweight/obesity and inflammatory conditions in children, and adults. These salivary biomarkers demonstrate moderate-to-strong correlation with serum biomarkers, in healthy as well as obese and diabetic individuals. Salivary markers of antioxidant status, including malondialdehyde and uric acid, show promise but will need to be explored further. While some studies show that salivary resistin and adiponectin concentrations are significantly correlated with serum values, and are known to be been elevated in obesity and diabetes, additional studies are needed to characterize such biomolecules in saliva and their relevance to inflammatory, metabolic, and cardiovascular conditions.

In conclusion, while saliva has the potential to become a premier diagnostic sample, substantial future research is required to standardize saliva collection techniques, validate salivary biomarkers of inflammation and insulin-resistance, across various life-stages and conditions, and establish reference ranges, before it can be used as a diagnostic fluid for cardiometabolic risk assessment.

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TOPIC HIGHLIGHT

WJD 5th Anniversary Special Issues (1): Insulin

B7-H4 as a protective shield for pancreatic islet beta cells

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Abstract

Auto- and alloreactive T cells are major culprits that damage β-cells in type 1 diabetes (T1D) and islet transplantation. Current immunosuppressive drugs can alleviate immune-mediated attacks on islets. T cell co-stimulation blockade has shown great promise in autoimmunity and transplantation as it solely targets activated T cells, and therefore avoids toxicity of current immunosuppressive drugs. An attractive approach is offered by the newly-identified negative T cell co-signaling molecule B7-H4 which is expressed in normal human islets, and its expression co-localizes with insulin. A concomitant decrease in B7-H4/insulin co-localization is observed in human type 1 diabetic islets. B7-H4 may play protective roles in the pancreatic islets, preserving their function and survival. In this review we outline the protective effect of B7-H4 in the contexts of T1D, islet cell transplantation, and potentially type 2 diabetes. Current evidence offers encouraging data regarding the role of B7-H4 in reversal of autoimmune diabetes and donor-specific islet allograft tolerance. Additionally, unique expression of B7-H4 may serve as a potential biomarker for the development of T1D. Future studies should continue to focus on the islet-specific effects of B7-H4 with emphasis on mechanistic pathways in order to promote B7-H4 as a potential therapy and cure for T1D.

Key words: Diabetes mellitus; Autoimmunity; Transplantation; Co-stimulation blockade; Biomarker

Core tip: Onset of type 1 diabetes is driven by defects in immune regulation, resulting in β-cell autoimmunity. However, there may be mechanisms inherent to the β-cell that may prevent or slow development of autoimmunity and progression of disease. One such factor is B7-H4, which acts at the islet-immune interface to defend β-cells from autoimmune diabetes and to protect transplanted islet allografts.

Sun AC, Ou D, Luciani DS, Warnock GL. B7-H4 as a protective shield for pancreatic islet beta cells. *World J Diabetes* 2014; 5(6): 739-746 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/739.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.739

INTRODUCTION

Pathophysiology of diabetes, current therapies and their limitations

Diabetes mellitus affects 382 million people world-wide today, and this number is expected to increase by 55% by 2035[1]. Diabetes is a chronic metabolic disease which stems from insufficient production of insulin by pancreatic β-cells and/or inability of the body to respond to insulin. There are two major forms of diabetes-type 1 diabetes (T1D), and type 2 diabetes (T2D). While differing in their pathogenesis, both types of diabetes result from failure and/or loss of insulin-producing β-cells that eventually translate to a state of chronic hypergly-

cemia[2-4]. Persistently high blood glucose concentrations are associated with this disease, which result in both acute metabolic conditions such as diabetes ketoacidosis and long-term vascular complications such as diabetic retinopathy, nephropathy, and neuropathy[2,4,5]. These devastating complications lead to enormous socioeconomic burdens, mandating a pressing need to find a cure.

There are both differences and similarities in mechanisms by which β-cell injuries occur in T1D and T2D. T1D has been identified as an autoimmune disease in which insulin-producing β-cells are destroyed by targeted immune attack in genetically susceptible individuals. It is believed that environmental events initially trigger the recruitment of $CD4^+$ and $CD8^+$ T cells to the islets of Langerhans and mount continuous attacks against autoantigens on β-cells, resulting in β-cell death[4,5]. T2D, closely linked to aging and obesity as well as a certain level of genetic susceptibility, is characterized by insulin insensitivity due to insulin resistance in peripheral tissues, which leads to β-cell stress[4,6,7]. T1D and T2D overlap in β-cell stress and death pathways despite differences in initiating triggers[3]. One such common pathway is endoplasmic reticulum (ER) stress, which can activate downstream signaling cascades collectively known as the unfolded protein response (UPR)[3]. Various conditions such as nutrient deprivation, inflammation, alterations in oxidation-reduction balance and elevated levels of glucose and lipids can all lead to accumulation of unfolded proteins in the ER lumen. In response to this ER stress, the UPR serves as a compensatory mechanism to restore ER homeostasis by increasing the protein folding capacity of the ER and muting protein translation[8-10]. However, chronic ER stress can shift the UPR towards a pro-apoptotic state[8,9]. In T2D, increased demand on insulin production due to progressive insulin resistance, combined with exposure to increased levels of glucose and fatty acids, induces prolonged β-cell ER stress, thus triggering cell death *via* apoptotic pathways[6,7,11]. Growing evidence also implicates ER stress as one of the factors that contribute to T1D[8,12,13]. Pro-inflammatory cytokines secreted by infiltrating immune cells in the islets of T1D patients could induce apoptosis *via* signal transducers such as STAT-1 and nuclear factor-kappa B[3,14,15], and cytokines could also negatively impact ER homeostasis and cause UPR dysregulation, which contributes to β-cell demise[3,16,17]. Knowledge of overlapping β-cell injury mechanisms between T1D and T2D can provide valuable insight into pathogenesis of diabetes, guiding rational development of therapeutics that target instigators of both T1D and T2D.

Treatments for diabetes have been designed to address glycemic control and alleviate diabetic complications. Depending on the severity of insulin resistance, management of T2D can be achieved through lifestyle and diet modifications. Commonly used pharmacological agents for T2D include insulin sensitizers, insulin secretagogues, incretin-based therapies, and insulin analogues[11]. Most T1D patients still rely on exogenous insulin injection to maintain euglycemia. However, stringent monitoring of blood glucose level is needed and the use of exogenous insulin carries the risk of hypoglycemic episodes that can be life-threatening.

In search of the elusive "cure" of diabetes, it would be desirable to halt the autoimmune attacks on β-cells, or to prevent it altogether. Current on-going clinical trials for T1D are focusing on using immunomodulation strategies to delay disease onset and preserve β-cell function in full blown diabetes. Examples of these drugs include anti-CD3 (teplizumab) and anti-CD28 (rituximab), antibodies to inhibit autoreactive T cells and B cells. CTLA4-Ig (abatacept), an inhibitory molecule for T cells, also showed promise in previous clinical trials to prolong insulin production in newly-diagnosed T1D patients[18].

Transplantation of insulin-producing tissue also provides a therapeutic option for diabetes. Whole pancreas transplantation yields better glycemic control compared with insulin injections, but subjects patients to major surgery with associated risks, and is therefore only offered to patients with severe diabetic complications. Islet cell transplantation is a relatively safe and fast alternative, in which islets isolated from cadaveric donors are infused into the liver *via* the hepatic portal vein[19,20]. With the development of the Edmonton Protocol, islet cell transplantation has become a reproducible, standardized procedure in multiple medical centers around the world which improves glycemic control[19,21]. Patients who received islet cell transplantation also showed markedly reduced diabetic retinopathy and nephropathy compared with patients who were treated with conventional medical therapy[20,21]. Even though insulin independence declined during prolonged follow up, partial graft function was maintained in 80% of the patients, as measured by C-peptide secretion[21]. Despite ongoing improvements in islet transplantation, eventual graft dysfunction, failure, and rejection remain a challenge[19,20].

The limited success of β-cell protection in various studies has attracted interest to novel β-cell immunoprotective strategies. In the following we review recent findings that suggest the negative co-stimulatory molecule B7-H4 has unique functions in the pancreatic islets that carries the potential to act as not only as a natural but also a therapeutic "shield" for β-cells during the development of diabetes and following pancreatic islet transplantation, as well its prospective role as a novel biomarker for T1D.

B7-H4: A NOVEL IMMUNE-REGULATORY MOLECULE

B7-H4, also known as B7x, was identified in 2003, and belongs to the B7 family of immunoglobulins[22-24]. Genomic B7-H4 is encoded on the *VTCN1* gene, which is located on chromosome 1 and 3 in human and mouse, respectively[24]. Given that mouse and human share 87% amino acid identity, B7-H4 is a highly evolutionarily conserved molecule. Mature B7-H4 is a 50-80 kDa transmembrane protein consisting of one IgV and one IgC

region, which are encoded on exons Ⅲ, Ⅳ, and part of Ⅴ[22-24]. Like other members of the B7 family, it is up-regulated on the cell membrane of activated antigen presenting cells, and acts to modulate the immune response[22-24]. Upon binding to a putative yet unidentified counter-receptor on T cells, B7-H4 acts as a negative co-signaling molecule to inhibit T cell proliferation and cytokine production. One proposed mechanism of action is that B7-H4 arrests cell cycle progression of T cells at the G_0/G_1 phase[23]. Since T cell activation is dependent on the presence of co-stimulatory signals, the suppressive nature of B7-H4 highlights its therapeutic potential in autoimmune diseases.

Interestingly, B7-H4 exhibits a unique mRNA profile. Unlike other B7 molecules, B7-H4 mRNA is expressed in multiple peripheral tissues such as the spleen, lung, liver, and pancreas[23]. Protein expression of B7-H4 in peripheral tissues is minimal, and its role is subject of much debate[23,25,26]. It is possible that B7-H4 undergoes tight post-transcriptional or post-translational regulation that limits its protein expression in those tissues. It remains unclear what roles B7-H4 play in the periphery, and whether it has functions that are independent of its effect on T cells. We and others have shown that the pancreas expresses moderate level of B7-H4, especially in the endocrine cells[25,27]. This raises the question of what the specific functions of B7-H4 are in pancreatic islets, and suggests the intriguing possibility that activity of B7-H4 is not limited to immune-modulation. For the purpose of this review, we will focus on the existing evidence which indicates that B7-H4 plays an essential role in islet autoimmunity and islet allotransplantation, and report data from cancer studies which alludes to other non-immune functions of B7-H4. All of the roles, known and potential, are shown in Table 1, which are classified as autoimmunity modulator, allograft protection, UPR modulation, and biomarker of β-cell immunity. This manuscript extends beyond previous reviews of B7-H4 by highlighting the importance of endogenous B7-H4 expression in β-cells, suggesting that the B7-H4 pathway for treating T1D may be more advantageous than other co-stimulatory molecules.

B7-H4 AS A PROTECTIVE SHIELD FOR β-CELLS IN T1D

Regulation of autoreactive T cells in autoimmune diseases can be achieved through various methods, such as regulatory T cell (Treg) therapy, interleukin (IL)-2 pathway manipulation, tolerance induction with antigen administration, and co-stimulation blockade[28]. As a negative co-signaling molecule, B7-H4 has the potential to down-regulate autoreactivity in autoimmune diseases such as T1D. While B7-H4 deficiency itself does not cause autoimmune diseases, various studies showed that B7-H4 plays an important role in inhibition of auto-reactive T cells in diseases such as experimental autoimmune encephalomyelitis, and rheumatoid arthritis[22,27]. Genome-wide association studies have also uncovered certain Single Nucleotide Polymorphisms within the B7-H4-encoding *VTCN1* gene as disease-causing in the context of diabetes, further implicating B7-H4 as a potential regulator of T1D[29].

Immunosuppressive functions of B7-H4 was confirmed in experimental T1D models using B7-H4-immunoglobulin (B7-H4 Ig), a recombinant protein derived from fusion of the immunoglobulin constant region to the extracellular domain of B7-H4[30,31]. Both intraperitoneal injections of B7-H4 Ig and cell-associated B7-H4 inhibited proliferation and cytotoxicity of $CD4^+$ and $CD8^+$ T cells *in vitro*[22-24,32]. Juvenile NOD mice treated with B7-H4 Ig exhibited significantly later onset as well as reduced incidence of diabetes[31]. This coincided with a reduction in proliferation and activation of both $CD4^+$ and $CD8^+$ subsets of T cells in the islet infiltrates[31]. In support of this, our preliminary findings suggested that β-cell specific over-expression of B7-H4 in transgenic NOD mice significantly decreased T1D incidence compared with wild type NOD mice (unpublished data). In conjunction with its preventive role in the onset of autoimmune diabetes, B7-H4 reversed incidence of established T1D. Return of glycemic control was observed in newly-onset diabetic NOD mice following B7-H4 Ig injections[33]. Conversely, adoptive transfer of diabetogenic T cells into B7-H4 deficient mice resulted in more exacerbated disease than wild-type controls[27]. It was hypothesized that B7-H4 did not have an effect on recruitment of immune infiltrates during the pre-diabetic stage, but rather, it prevented the progression of insulitis to overt diabetes by arresting severe insulitis at 12 wk of age in NOD mice[27,31]. This modulation of immune status at later stage of disease may be associated with down-regulation of the Th1 cells, which are widely accepted as key mediators of autoimmune diseases[31].

Mechanistic studies examining the role of B7-H4 showed that it was able to limit autoreactive CTLs, and suppressed secretion of inflammatory cytokines in the periphery[33]. For instance, levels of Th17-associated cytokines, IL-6, and IL-23, were reduced in B7-H4 treated animals[33]. This reduction was concomitant with a decrease in Th17 cells, a subpopulation of $CD4^+$ T cells that produce IL-17, IL-17F, IL-21, and IL-22, and have been implicated in various autoimmune conditions[34,35]. IL-17 is an inflammatory cytokine that may stimulate the production of other inflammatory cytokines, and is present at high levels in autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis[36-38]. Importantly, elevated Th17 cells were found in NOD mice as well as T1D patients, and were suggested to be a contributing factor to the pathogenesis of autoimmune diabetes[39-41]. One mechanism by which Th17 cells were proposed to act in T1D patients was to cause a disturbance in the ratio of T effective cell (Teff)/Treg cells, which shifted the adaptive immune response to allow development of T1D[42]. Additionally, Th17 cells were able to convert to a Th1 phenotype and stimulated cytotoxic T lymphocytes (CTL) to further contribute to autoimmunity[39]. Consistent with roles of B7-H4 in islet

Table 1 Evidence for immune regulatory and β-cell autonomous roles of B7-H4 in experimental/human diabetes

Role	Model	Summary of findings	Application	Ref.
Autoimmune modulator	NOD mouse	B7-H4 Ig inhibits development of, and reverses newly-onset autoimmune diabetes	Prevents/ reverses T1D	[31,33]
Allograft protection	NIT cell line	B7-H4 transfected NIT cells promote β-cell allograft survival	Suppresses islet graft rejection	[44]
	Mouse	Adenoviral-transduced B7-H4 donor islets enhanced islet allograft survival, and promotes donor-specific tolerance		[43,46]
	Mouse	B7-H4 transgenic islets improve islet allograft survival	Preserves β-cell mass in T1D/T2D	[51]
Non-immune dependent UPR and cell survival regulator	Pancreatic carcinoma-derived cell lines	B7-H4 knock-down increases cell apoptosis		[56]
	Renal carcinoma tissues and cancer cell lines	Human intracellular B7-H4 is identified as a cytoplasmic-nuclear shuttling protein that contains a NLS		[57]
	Mouse	B7-H4 modulates UPR in isolated pancreatic β-cells		Unpublished
Biomarkers of β-cell immunity	Mouse	B7-H4 RSS0.2 mRNA splice form is correlated with different stages of T1D	Detects β-cell autoimmunity	Unpublished
	Human	Reduced B7-H4 expression and B7-H4/insulin colocalization is detected in pancreata of T1D patients		[25]
	Human	Elevated sB7-H4 is present in RA and newly-onset T1D patients		[61,62]

T1D: Type 1 diabetes; NOD: Non-obese diabetic; UPR: Unfolded protein response; NLS: Nuclear localization signal.

autoimmunity, pancreata of B7-H4 deficient mice expressed significantly enhanced production of IL-17 and interferon (IFN)-γ, while islet-specific over-expression of B7-H4 led to a dramatic reduction in IL-17 and IFN-γ[27]. *In vitro* studies showed that cultured splenocytes displayed less affinity toward a Th17 phenotype when incubated with B7-H4 Ig, and sequestering of B7-H4 restored Th17 polarization[33]. This effect was dependent on increased IFN-γ production by the splenocytes, suggesting that inhibitory effect of B7-H4 on Th17 cell differentiation was due to stimulation of IFN-γ release[27,33]. However, it seemed that inhibition of Th17 cells by B7-H4 did not shift the Teff/Treg ratio towards Teff cells, neither did it act to expand the Th2 cell population, which is classically known as the anti-inflammatory T cell phenotype[27]. It is possible that the reduction in Th17 cells may potentially reduce the pathogenic Th1 phenotype that contributes to autoimmunity.

In summary, B7-H4 has been demonstrated to have functionality in both arresting and reversing newly-onset T1D in rodent models, and thus shows great promise as a preventative measure and a potential treatment for the disease. Current evidence suggests that B7-H4 prevents progression of severe insulitis to overt diabetes, in part, by suppressing mediators of autoimmunity such as Th1 and Th17 cells. Further research will help clarify the upstream signaling events leading to the observed beneficial effects and may significantly advance our ability to harness the potential of B7-H4 as a therapeutic for T1D.

B7-H4 INDUCES DONOR SPECIFIC TOLERANCE IN ISLET TRANSPLANTATION

B7-H4 also promotes the viability of islet grafts, and thus has significant potential for improving clinical islet transplantation as a treatment for diabetes[43-46]. Transplanted islets face many overlapping forces that conspire to limit graft function and survival, ranging from mechanical stress during isolation procedures to adverse effects of immunosuppressive drugs post-transplantation. During islet isolation and transplantation, conditions of hypoxia and nutrient deprivation collectively induce oxidative stress, ER stress and apoptosis, resulting in a decline in functional β-cell mass[47]. In the case of T1D patients, islet grafts not only encounter autoimmune surveillance, but also experience rejection mediated by alloreactive T cells. This process occurs due to priming of $CD4^+$ T cells by alloantigens presented by MHC molecules on antigen presenting cells. Activated $CD4^+$ T cells then promote the differentiation and proliferation of $CD8^+$ T cells, which attack the donor tissue. Current immunosuppressive regimens for islet transplant recipients consist mostly of tacrolimus (FK506), sirolimus (rapamycin), and mycophenolate mofetil (MMF)[20,21,48]. Generalized side effects of these drugs include increased risks for infection and malignancy, hypertension, lung toxicity, and cardiac damage. Tacrolimus has been linked to nephrotoxicity, which can be especially damaging to recipients who are at risk for diabetic nephropathy[19]. Importantly, studies have demonstrated that these drugs induced islet cell apoptosis and impaired islet function based on their mechanisms of action[19,49]. For instance, tacrolimus and sirolimus inhibit calcineurin and mammalian target of rapamycin, both of which are involved in insulin signaling and secretion[49,50]. It is therefore critical to identify novel therapeutics that offers immune-protection with minimal level of toxicity and side effects. B7-H4 is a molecule which can suppress autoimmunity as well as modulating alloreactivity, which makes it a perfect candidate for islet cell transplantation especially in T1D patients[45].

Initial investigation into the role of B7-H4 on allograft rejection demonstrated that B7-H4 protected NIT cells, a functional NOD-derived β-cell line, from injury[44]. Survival of NIT cells allotransplanted into diabetic mice was prolonged by B7-H4 transfection[44]. This was associated with reduced proliferation of recipient splenocytes,

decreased production of IFN-γ, and increased Tregs in the spleen[44]. The protective effect of B7-H4 in allotransplantation was further observed in B7-H4 adenoviral-transduced islets and B7-H4 transgenic islets. Local overexpression of recombinant B7-H4 adenovirus (Ad)-B7-H4 in intact mouse islets preserved original β-cell function and endogenous glucose responsiveness at both basal and high glucose conditions[43]. Furthermore, mice who received islets transduced with (Ad)-B7-H4 demonstrated longer allograft survival with significantly reduced infiltrates compared with control recipients[43]. Elevated Tregs and reduced cytotoxic T cells were observed in transduced islet grafts, further suggesting that B7-H4 may alter the immune environment at the graft site to induce tolerance[43]. Similarly, B7-H4 transgenic islets promoted islet allograft survival, concurrent with migration of Tregs to the graft site[51]. Tregs are known to secrete IL-10, an anti-inflammatory cytokine, and can also induce IL-10 secretion in APCs[52]. IL-10 suppresses Th1 phenotype, thus inhibiting Th1 effector cells such as $CD8^+$ T cells. In addition, Tregs also stimulated B7-H4 expression on monocytes and other APCS[52], which may act as negative co-signals to restrain T cell reactivity against donor antigens. These studies demonstrated that allotransplantation outcomes can be largely influenced by T cell co-signaling molecules, where Tregs played an important role in B7-H4 induced tolerance.

Interestingly, B7-H4 is able to achieve donor-specific tolerance rather than general unresponsiveness towards foreign antigens. When the primary B7-H4-transduced islet graft was removed and replaced with a secondary graft from the same donor mouse strain, graft survival was higher compared with a secondary graft from a third-party donor strain[46]. Isolated splenic leukocytes from recipient mice showed decreased IL-2 levels due to reduced number of IL-2 secreting cells[46]. However, no differences were observed in Tregs between mice that received same donor strain islets compared with those transplanted with third party strain islets[46]. It is possible that while Tregs are central to establishment of allograft tolerance, they may not be the main contributors to the maintenance of the secondary graft. Conceivably, B7-H4 can act on other pathways to affect IL-2 secretion and induction of donor-specific tolerance, however, this avenue of research is yet to be explored.

B7-H4 AS A DIRECT MODULATOR OF THE UNFOLDED PROTEIN RESPONSE AND CELL DEATH

The ubiquitous expression of B7-H4 in peripheral tissues has led to speculations regarding its role independent of the immune system. In support of this, studies on cancer cells reported elevated expression of B7-H4 in the cytoplasm and cell membranes from breast, uterus, and pancreas cancer cells[53-55], and its expression was correlated with tumor progression. It has been speculated that up-regulation of B7-H4 may help cancer cells evade immunosurveillance as well as being a direct tumorigenic factor independent of the immune system[56,57]. Consistent with these hypotheses, Zhang *et al*[57] demonstrated that human B7-H4 contains a nuclear localization sequence that allows B7-H4 to shuttle between the cytoplasm and the nucleus, and may regulate transcription of genes involved in cell apoptosis. Qian *et al*[56] also showed *in vitro* B7-H4 gene silencing in pancreatic cancer cells led to reduced proliferation rate and an increase in cell apoptosis that correlated with increased expression of the pro-apoptotic Bax protein and caspase activation. B7-H4 may thus play a central role in survival and apoptosis, but the exact mechanisms by which it facilitates disease progression remain an area of active investigation.

Specifically in the β-cells, endogenous B7-H4 may regulate stress *via* other cell-autonomous signaling pathways. Data from our lab suggested that *in vivo* administration of B7-H4 Ig affected the age-dependent expression of key UPR genes in the islets of NOD mice (unpublished). Notably, additional *in vitro* experiments on islets from transgenic islets with β-cell specific B7-H4 expression suggested that B7-H4 can modulate β-cell UPR signaling and may thus affect the ability of pancreatic islets to adapt to ER stress (unpublished data). In conjunction with the evidence from tumor cells, these findings support the intriguing possibility that B7-H4 also has non-immune-mediated roles in maintaining β-cell function and survival, and highlight promising new avenues for future research.

SPECIFIC EXPRESSION OF B7-H4 AS A POTENTIAL NOVEL BIOMARKER FOR T1D

While the end result of T1D is significant loss of islet β-cells that warrants the need for life-long insulin replacement, progression to end-stage diabetes occurs in several stages[58,59]. The initial step is development of islet autoimmunity, which manifests as presentation of autoantibodies to putative antigens such as GAD, ZnT8, IA-2, and insulin. Measurements of these autoantibodies have proven useful for predicting diabetes. However, after the initiation of islet autoimmunity, they are no longer able to offer consistent information regarding disease progression. From the time of autoimmunity onset to clinical diabetes there is a relatively long pre-diabetic stage. This is a critical time for therapeutic intervention, as there is theoretically still adequate functional β-cell mass at this stage of dysglycemia to preserve sufficient endogenous insulin secretion that obviates full blown T1D[60]. It is therefore vital to develop reliable markers for monitoring β-cell loss and characterizing each stage of T1D in order to determine the efficacy of therapeutic interventions associated with each stage.

In the prediction of autoimmunity, B7-H4 has been proposed to serve as a candidate biomarker for rheumatoid arthritis (RA)[55,61]. Serum samples indicated that levels of soluble B7-H4 protein (sB7-H4) in patients diagnosed with RA were significantly higher than those in

healthy donors[61]. In addition, elevated levels of sB7-H4 were associated with increased disease severity[61]. Our results showed a trend of higher sB7-H4 in diabetic children, though not statistically significant. This data agreed with a more recent study, which confirmed that sB7-H4 were elevated in newly-onset T1D patients[62]. Previous characterization of the B7-H4 gene using human multiple cDNA panels demonstrated that there are two major versions of B7-H4 transcripts from the pancreas tissue: A full-length (2.0 kb) transcript which is shared with other organs, and a shorter (1.2 kb) transcript version which is specific for pancreas[23,24]. We have also detected the presence of an additional 0.2 kb B7-H4 mRNA splicing species (RSS0.2) in the serum of T1D patients (unpublished data). Moreover, preliminary studies showed that high levels of circulating B7-H4 RSS0.2 were correlated with newly-onset T1D (< 1 year), while intermediate levels of this mRNA splice form were observed in patients with longer-term disease (1 year), and the lowest levels were found in patients with late stage T1D (2-5 years). This suggests that sB7-H4 and unique B7-H4 splice forms may serve as a novel biomarker for determining various stages of T1D.

In the human pancreas B7-H4 is more abundantly expressed in the islets than the exocrine tissue at both mRNA and protein level[25,27]. Recently, Cheung *et al*[25] showed that altered B7-H4 expression occurred in T1D and insulinoma. Multi-fluorescence immunohistochemical analyses revealed moderate expression of B7-H4 in non-diabetic pancreatic islets, significantly reduced protein expression in T1D islets, and high expression in insulinoma tumor cells[25]. Furthermore, correlation analyses demonstrated B7-H4 co-localization with insulin in both human and mouse islet[25,27]. Interestingly, the B7-H4/insulin co-localization was dramatically reduced in both T1D islets and insulinomas compared with non-diabetic islets[25]. It is possible that the reduced association between B7-H4 and insulin may reflect diseased islet states, agreeing with the observation that B7-H4 protein and mRNA expressions in islet β-cells and in sera may be useful as indicators of islet dysfunction and β-cell death/loss in the progression of T1D.

CONCLUSION

B7-H4 is the newly-identified member of the B7 immunoglobulin family commonly associated with co-stimulatory or inhibitory signals for T cells. Even though the putative receptor for B7-H4 on activated T cell is yet to be identified, its marked ability to suppress and reverse autoimmune diabetes has been demonstrated in various cellular and animal models. Furthermore, B7-H4 can induce donor-specific tolerance in islet allografts, which holds great promise as an adjunct for modern paradigms of immunosuppression. In the pancreas a relative abundance of B7-H4 in β-cells alludes to novel functions in the pancreatic islets, and ongoing work hints at important roles of endogenous B7-H4 for β-cell health and function. Of note, B7-H4 also displays a unique expression profile unlike that of other B7 family members, and variations in its protein and mRNA splicing species may act as potential biomarkers for T1D. Further research into both the immune-regulatory and β-cell-autonomous roles of B7-H4 promises to elucidate its contributions to β-cell health and survival, thus identifying it as a novel β-cell protective shield for patients suffering from diabetes.

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TOPIC HIGHLIGHT

WJD 5th Anniversary Special Issues (4): Diabetes-related complications

Is the present cut-point to define type 2 diabetes appropriate in Latin-Americans?

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Abstract

The diagnosis of diabetes mellitus type 2 (DM2) is based either on increased plasma glucose or Glycated hemoglobin levels. Since these measures are the only means for diagnosis of DM2, they must be well adapted to each population according to their metabolic characteristics, given that these may vary in each population. The World Health Organization (WHO) determined the cut-points of plasma glucose levels for the diagnosis of DM2 by associating hyperglycemia with the risk of a specific microvascular complication-retinopathy. Cardiovascular diseases are however the principal causes of mortality in patients with DM2 and we reported that in the Colombo-Ecuadorian population impaired fasting glucose and impaired glucose tolerance are both risk markers for myocardial infarction. We propose that the current cut-points accepted by the WHO need to be revaluated in populations such as Latin America and that there should be lower cut points for glycaemia in this population, to reduce the prevalence of cardiovascular complications associated with DM2.

Key words: Type 2 diabetes; Cut-off points; Cardiovascular diseases; Plasma glucose; Coronary disease

Core tip: We propose that the current cut-points to define type 2 diabetes accepted by the World Health Organization need to be revaluated in populations such as the Latin America and that there should be lower cut points for glycaemia in this population, to reduce the prevalence of cardiovascular complications associated with diabetes mellitus type 2.

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INTRODUCTION

The World Health Organization (WHO) issued technical reports relating to diabetes in the years 1965[1], 1980[2], 1985[3], and 1999[4]. Over this period, there have been significant changes in the diagnostic criteria and for the classification of diabetes mellitus (DM) and intermediate hyperglycemia[5], also known as dysglycemia or prediabetes. In the first report in 1965, the WHO set a DM cut-off of $\geqslant$ 130 mg/dL according to the patient's response to a two hour oral glucose tolerance test (OGTT) and

their clinical manifestations[1]. Then in 1980, specific criteria were introduced, such as retinopathy or the presence of glucose in urine, or a random plasma glucose tests of ⩾ 200 mg/dL, and values for Fasting Plasma Glucose (FPG) of ⩾ 145 mg/dL or glucose in venous plasma 2-h after glucose load (75 g) ⩾ 200 mg/dL for the diagnosis of DM[2]. In 1985, the cut-off points for FPG were decreased to ⩾ 140 mg/dL while the OGTT of ⩾ 200 mg/dL was maintained[3].

In 1997, The Expert Committee of the American Diabetes Association (ADA) released their new recommendations for the classification and diagnosis of diabetes. The stage impaired glucose tolerance (IGT) was retained but there were several major changes including: (1) the preferred use of the terms "type 1" and "type 2" instead of "insulin-dependent" and "non-insulin-dependent" to designate the two major types of DM; (2) The analogous intermediate stage of fasting glucose was named "impaired fasting glucose (IFG)"; and (3) a lower cutoff for FPG from ⩾ 140 mg/dL to ⩾ 126 mg/dL to diagnose diabetes was established (this level of FPG having been found equivalent to the 200 mg/dL value in the oral glucose tolerance diagnostic test)[5].

In 1999, the WHO then amended the cut-off points to ⩾ 126 mg/dL in fasting glucose and maintained the ⩾ 200 mg/dL for OGTT, which was established in 1980. The new fasting criterion was chosen to represent a value at the upper end of the range, which in many patients corresponds to the diagnostic significance of the 2-h post-load concentration, which was not modified[4].

The criteria currently used for the diagnosis of diabetes and intermediate hyperglycemia have been in place globally for almost a decade, and are widely accepted by the ADA[6] and the WHO[7,8] using the four following criteria: Symptoms of hyperglycemia such as polyuria, polydipsia, and unexplained weight loss, and a casual plasma glucose ⩾ 200 mg/dL; casual-defined as a result obtained at any time of the day; (2) A 2-h plasma glucose ⩾ 200 mg/dL during an OGTT. This test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water; (3) Fasting glycemia levels ⩾ 126 mg/dL; and (4) Glycated Hemoglobin (HbA1c) ⩾ 6.5%. Both the ADA and the WHO believe that sufficiently stringent quality assurance tests are in place and that assays are standardized to criteria aligned to the international reference values, so that there are no conditions present which preclude an accurate measurement of HbA1c.

HOW WERE THE CUT-OFF POINTS FOR DM DETERMINED?

While plasma glucose and HbA1c represent the basic criterion measures to define DM, the universally utility of these determinations has been questioned[9]. The diagnostic cut-off points for diabetes were based on two sets of evidence: (1) Plasma glucose levels associated with an increased risk of specific microvascular complications, particularly retinopathy; and (2) The distribution of plasma glucose in the general population[9-11].

However, there are a number of methodological weaknesses of the studies that have reported the cut-points for increased risk of retinopathy including inadequate statistical power for this type of analysis[10]. Moreover, these studies used different methods to diagnose retinopathy and some used patients already identified as diabetic, while others used non-diabetic patients[10,11]. In addition, some reports included people with diagnosed DM who were receiving blood glucose lowering treatment introducing a bias associated with treatment-induced effects on plasma glucose. Excluding people with treated diabetes from analyses eliminates the bias related to the treatment effect, but changes the characteristics of the diabetic population[12].

One of the most important studies to support the cut-points was conducted by Ito *et al*[11], which included 12.208 people and began in 1965 and lasted until 1997. The authors reported a significantly increased prevalence of retinopathy at a baseline FPG cut-point of 125 mg/dL and 198 mg/dL in 2-h post-glucose load.

Other microvascular complications are more weakly associated with plasma glucose levels than retinopathy[13]. Studies which have examined the relationship between plasma glucose and proteinuria, reported a significant association but weaker than with retinopathy[13]. For instance, among patients with DM, only 20%-40% of patients with microalbuminuria will progress to overt nephropathy, and only 20% will go on to end-stage renal disease within the next 20 years[14]. Moreover, the data showing a relationship between plasma glucose and biopsy confirmed diabetic renal disease is not totally convincing, since the prevalence of non-diabetic nephropathy in the patients with DM who underwent renal biopsy varies from 10% to 85% in different reports[15]. Furthermore, FPG and HbA1c values associated with the presence of diabetic nephropathy were exceptionally high: 183 ± 61.9 mg/dL and 8.6% ± 2.4%, respectively[16].

The distribution of plasma glucose in the general population was another source of data used to define cut-points. In 2006, the WHO reported that the distribution of plasma glucose among the population was either unimodal, in which the entire population is represented by a single curve, or bimodal, represented by two overlapping curves[7]. However, an analysis of DETECT-2, representing plasma glucose data measured during an OGTT in 26 different countries, found a wide variation in cut-points[9]. Cut-points for FPG in different countries ranged from 103 to 153 mg/dL (median 128.5 mg/dL), and for 2-h plasma glucose from 164.7 to 323.9 mg/dL (median 224.4 mg/dL). Moreover, when known diabetes was removed from the analysis, the distributions of plasma glucose do not generally give rise to a bimodal structure that is useful for deriving a cut point for diabetes. Thus, bimodality seems not to be a suitable method for defining diagnostic cut points for diabetes in population studies which include people of different origin[9].

Bimodal distribution has also been reported in a

number of populations with a high prevalence of diabetes, including the American Pima Indian, Micronesian of Nauru, Egyptian, Mexican, Papua New Guinea, and South African populations[9,17]; while few studies on bimodality have been conducted in populations with a low prevalence of diabetes[18].

Recently, and in support of the use of HbA1c as a diagnostic criterion, several studies have noted that HbA1c reflects average plasma glucose and does not require any special preparation such as fasting. These features led to it becoming the gold standard for assessing glycemic control in people with diabetes, and it has also become a means to assess glucose tolerance in those with undiagnosed diabetes[12]. The relationship between HbA1c and the presence of retinopathy is similar to that of plasma glucose, making it at least as accurate in defining the level of hyperglycemia at which retinopathy prevalence increases[19].

Moreover, HbA1c has appreciable superior technical attributes, including less pre analytic instability and biological variability, and is a more clinically convenient measure. HbA1c has been demonstrated to be more reliable than FPG, with a day to day coefficient of variation of less than 2% compared to 16% for FPG[20].

Studies have now established an HbA1c level associated with an increase in the prevalence of moderate retinopathy, providing strong justification for assigning an HbA1c cut-off point of $\geqslant$ 6.5% for the diagnosis of diabetes[8]. Although this cut-off point must not be used as an absolute dividing line between normal glycemia and diabetes, this value is sufficiently sensitive and specific to identify individuals who are at risk of developing retinopathy and who therefore, should be diagnosed as diabetic[20].

HbA1c however does have some limitations which should be considered when using it as criteria for the diagnoses of DM. First, the cost of the test precludes its routine use. Second, there are some specific conditions that can influence and therefore preclude HbA1c testing, including the following hemoglobin traits: HbS, HbC, HbF, and HbE, as well as various types of anemias, pregnancy, uremia and blood transfusions[21]. Some of these factors may represent an additional problem in under-resourced countries, due to their higher prevalence of anemia and hemoglobinopathies[21]. Moreover, it should be noted that there are normal age-related increases in HbA1c[22].

PROPOSED MECHANISMS TO EXPLAIN THE NEGATIVE EFFECTS OF HYPERGLYCEMIA ON THE VASCULAR WALL

Blood glucose level can also be a risk marker for cardiovascular diseases (CVD) among apparently healthy non-diabetic individuals[23-26]. The effects of elevated glycemia levels include non-enzymatic glycosylation of proteins, increased metabolism of glucose through the polyol and glucosamine pathways and the generation of free radicals[27-32]. Glycosylation of low-density lipoprotein makes it more susceptible to oxidization and therefore more atherogenic[27]. Advanced glycosylation end products (AGEs) can cross-link proteins, particularly in the extracellular matrix of the vascular wall[31,32]. Metabolism of excess glucose by secondary pathways can also alter cell function by modifying signal transduction and changing the oxidative potential of cells[30]. This may contribute to general cell damage and dysfunction[28]. These pathways can also activate tissue-specific protein kinase C[29] and increase in the activity of which decreases fibrinolysis and nitric oxide (NO) levels and increases cell proliferation and coagulation, contributing to the progression of CVD[28-30].

The association between intermediate hyperglycemia and coronary heart disease has been explained by the predisposition of these subjects to subsequently present DM2, a condition that as noted above, is directly related to the development of CVD[27]. However, hyperglycemia *per se* may also be directly involved in the development of atherosclerosis by promoting metabolic and structural changes in the endothelium that eventually produce irreversible damage. Therefore, the association between hyperglycemia and cardiovascular risk should be considered as a continuum, rather than one that depends only on reaching a specific cut point.

Experimental studies suggest that hyperglycemia reduces the activity of NO at the vascular endothelial level[28]. Hyperglycemia induces a series of cellular events that increase the production of reactive oxygen species that inactivate NO and lead to the formation of peroxynitrite[29,30]. In addition, mitochondrial production of reactive oxygen species increases the intracellular formation of AGEs[30], which affect endothelial function and activate the receptors for AGEs causing apoptosis and altered vascular structure[31-33]. In non-diabetic subjects, altered levels of post-load glucose have been associated with the presence of structural alterations at the level of the carotid arteries, manifested by increased carotid intima-media thickness[34-36]. Moreover, chronic hyperglycemia can also cause cellular structural changes, which would explain the known point of no return for the micro and macrovascular complications observed in diabetic patients[37-39]. Recent experimental studies with rats in which diabetes was induced using streptozotocin, demonstrated a loss of nitric oxide synthase function (NOS) in nitrergic neurons. This effect was mediated by an increased production of AGEs, oxidative stress and neuronal apoptosis, which was reversible only when treatment with insulin was introduced in early stages. After 12 wk of streptozotocin-induced diabetes, insulin therapy was not able to recover the function of the nitrergic neurons, which had suffered an increased apoptosis[37,38]. These experiments suggest that chronic hyperglycemia over time leads not only to an alteration of NOS function, but also in later stages to irreversible structural changes in different tissues. Since streptozotocin-induced DM is more similar to type 1 DM, it is therefore possible that the

underlying mechanism of vascular damage in type 2 DM is different to that described above. Nonetheless, this mechanism could be responsible for the development of atherosclerosis in the vascular wall of hyperglycemic patients. Thus, it is attractive to postulate that in the early stages of hyperglycemia, the use of hypoglycemic treatments could decrease the formation of AGEs, reversing endothelial dysfunction and preventing both structural disorder and the progression to CVD[39].

WHY SHOULD CUT POINTS OF PLASMA GLUCOSE TO DIAGNOSE DIABETES MELLITUS BE RE-EVALUATED?

We propose that CVD prevention depends on an early and aggressive intervention to control glycemia levels, probably at the prediabetes stage, to avoid reaching a "point of no return" with respect to structural alterations of the arterial walls. This proposal is supported by important clinical trials[40-44] such as the United Kingdom Prospective Diabetes Study which demonstrated that if an intensive treatment of hyperglycemia is started when DM2 is first diagnosed, there is a significant decrease in the number of cardiovascular events[41], maintained until 10 years after end of the study[40]. However, as recently demonstrated in clinical trials, if the intensive treatment is started after 8[42], 10[43], or 12[44] years of diagnosed DM2 the impact of the intensive treatment does not produce a decrease in the number of cardiovascular events (Table 1). These results highlight the importance of starting the hypoglycemic intervention earlier than is common practice currently.

The magnitude of the glycemia association with CVD risk has been reported in many studies[25,45], and although post-load blood glucose level has a linear relationship with CVD risk in the non-diabetic range, a possible threshold effect for FPG level appears to exist around 100 mg/dL[27]. There is an important body of information indicating that the cardiovascular risk starts at levels well below the cutoff point currently used for the diagnosis of DM2 and increases continuously[25,46]. Many studies show that non-diabetic patients with hyperglycemia have an increased risk of cardiovascular morbidity and mortality[46-51]. The meta-analysis of prospective studies conducted by Levitan *et al*[23] shows that the group with the highest post-load blood glucose level (midpoint range, 150-194 mg/dL) had a 27% greater relative risk (RR) for CVD compared with the group with the lowest level (midpoint range, 69-107 mg/dL) (RR = 1.27, 95%CI: 1.09-1.48).

Moreover, in a meta-analysis of studies that included a total of 95.783 people, Coutinho *et al*[25] found a linear relationship between glucose levels and subsequent cardiovascular events over a period of 12 years, reporting a RR = 1.33 (95%CI: 1.06-1.67) for those with FPG levels of 110 mg/dL and an RR of 1.58 (95%CI: 1.19-2.10) for patients with post-load blood glucose levels > 140 mg/dL.

The Whitehall Study[51] lasted 33 years and followed 17.869 male civil servants aged 40-64 years, of which 3.561 died of coronary diseases. In this study, the hazard of coronary mortality rose when 2-h blood glucose level reached 83 mg/dL (95%CI: 76-96). Between this level and 200 mg/dL, the age-adjusted hazard ratio was 3.62 (95%CI: 2.3-5.6). Although the data was applied at baseline in these male civil servants, this report has a limitation in that the findings are based on a 50 g OGTT, and a slightly differing dose-response relationship might be obtained with a 75 g glucose load.

The DECODE study[45] was a prospective European analysis of 22 cohorts with baseline glucose measurements for 29.714 subjects aged 30-89 who were followed-up for 11 years. After adjusting for other cardiovascular risk factors, the study reported an association between risk of death and both high glucose concentrations and very low glucose levels. Compared with a fasting plasma glucose of 81-110 mg/dL, the multivariate adjusted HR (95%CI:) for FPG < 81 mg/dL was 1.2 (1.0-1.4) for all causes, 1.3 (1.0-1.8) for CVD, and 1.1 (0.9-1.4) for non-cardiovascular mortality. For 2-h plasma glucose of 54.4-81 mg/dL, as compared with 2-h plasma glucose of 81.6-100 mg/dL the HRs were 1.1 (1.0-1.2) for all causes mortality, 1.1 (0.9-1.3) for cardiovascular mortality, and 1.1 (1.0-1.3) for non-cardiovascular mortality, respectively.

In the Asian Pacific Region, blood glucose data from 237.468 participants of 17 cohort studies are available[52]. Continuous positive associations were demonstrated between usual fasting glucose and the risks of cardiovascular diseases down to at least 88.6 mg/dL. Overall, each 18 mg/dL lower than usual fasting glucose was associated with a 21% (95%CI: 18%-24%) lower risk of total stroke, and 23% (95%CI: 19%-27%) lower risk of total ischemic heart disease. The associations were similar in men and women, across age-groups, and in Asian compared with Australasian (Australia and New Zealand) populations.

The China Heart Survey[53], a multicenter study, recruited 3.513 patients hospitalized for Coronary Artery Diseases (CAD), of whom 35.1% were admitted for acute CAD and 64.9% were elective admissions for CAD. At entry, 1.153 patients (32.8%) had known DM and 97 (2.7%) had newly diagnosed DM. Furthermore, 32.6% had IGT, and 4.7% had IFG. The proportion of patients with diagnosed DM increased from 32.8% at baseline to 52.9% post-OGTT analysis.

The GAMI study[54] of 181 patients admitted to two Swedish hospitals with acute myocardial infarction (AMI) and no history of DM, found a prevalence of 34% for prediabetes and 33% for de novo DM, leaving only 33% with no alteration in glucose metabolism. This distribution was similar when measurements were repeated at 3 and 12 mo. These findings were later confirmed by another study that included 4.961 patients with coronary disease enrolled in 110 centers throughout Europe[55]. In this study the prevalence of pre diabetes was 32% in those patients admitted with acute coronary syndrome and only 29% of enrolled patients had a normal carbohydrate metabolism.

Table 1 Differences in cardiovascular outcomes according to the time of disease (diabetes mellitus type 2) before the start of an intensive hypoglycemic intervention

Study	Time since diagnosis	Treatment	Mean outcomes
UKPDS 34 and 80[40,41]	Newly diagnosed	Metformin added to an experimental group, median glycated hemoglobin was 7.4% in the metformin group compared with 8.0% in the conventional group	↓ 32% for any diabetes-related endpoint ↓ 42% for diabetes-related death ↓ 36% for all-cause mortality A continued reduction in microvascular risk and risk reductions for myocardial infarction and death from any cause were observed during 10 yr of post-trial follow-up
The Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation trial[42]	7.9 yr	Gliclazide (modified release) plus other drugs as required to achieve a glycated hemoglobin value of 6.5% or less and Perindopril + Indapamide	No significant effects on major macrovascular events, death from cardiovascular causes, or death from any cause
The Action to Control Cardiovascular Risk in Diabetes trial[43]	10 yr	Individualized intensive therapy of a combination of any hypoglycemic drug targeting a glycated hemoglobin level below 6.0% or standard therapy targeting a level of 7% to 7.9%	The intensive-therapy group did not differ significantly from the standard-therapy group in the rate of the primary outcome (a composite of nonfatal myocardial infarction, nonfatal stroke, or death from cardiovascular causes) but had more deaths from any cause (primarily cardiovascular)
The Veterans Affairs Diabetes Trial[44]	11.5 yr	Intensive-therapy group goal was an absolute reduction of 1.5% in the glycated hemoglobin level, as compared with the standard-therapy group, metformin plus Glimepiride or Rosiglitazone	No significant effect on the rates of major cardiovascular events, death, or microvascular complications

UKPDS: United Kingdom Prospective Diabetes Study.

In Latin America, the ongoing multicenter Colombian-Ecuadorian study which includes until now 439 subjects distributed in 8 hospitals of Colombia and Ecuador to determine the prevalence of pre diabetes in patients with a first AMI shows that the combined prevalence of DM2 and prediabetes is 69.47%. Ninety subjects (20.50%) presented with antecedents of DM2; another 85 (19.36%) were diagnosed with DM2 while hospitalized; and 130 (29.61%) presented with prediabetes. Only 134 subjects (30.53%) were normoglycemic[56].

The existence of a strong association between cardiovascular risk factors and IFG has also been reported in Colombia, with an even greater association with the presence of abnormal plasma glucose levels after an oral glucose load[57]. Additionally, in our population there is evidence indicating that hyperglycemia is common in patients with already established coronary disease[58].

Furthermore, a Colombian population study found that an IFG > 100 mg/dL was the risk factor with the highest degree of association with the presence of CAD in patients with stable angina pectoris, independent of the presence of other traditional cardiovascular risk factors[58]. Moreover, in this population fasting hyperinsulinemia and the socio-economic status of individuals with a first myocardial infarction were the only factors that remained significant predictors of a new cardiovascular event after a multivariate analysis[59]. We have previously shown that Colombian people present a higher vulnerability to present with insulin resistance at lower levels of abdominal obesity in youth adults[60,61], in pregnancy[62], and in children[63].

Many years ago Hales and Barker demonstrated that low birth weight is associated with an increased risk of developing obesity, metabolic syndrome and DM2[64-66]. Based on the results of their pioneering work and subsequent confirmatory studies, we have proposed[67-69] that the fetal programming during pregnancy of women that have deficient nutrition and/or an increased frequency of subclinical infection and preeclampsia, have an increased risk of giving birth to a low birth weight child with a higher risk of subsequently developing insulin resistance (IR) and low degree inflammation. It is well established that children with low birth weight have a decreased mass of beta cells, nephrons, hepatocytes, and fewer muscle fibres. We recently demonstrated, in children and adolescents that low muscle strength is associated with increased adiposity, C-reactive protein, HOMA index and metabolic risk factors, and that this association was stronger in with low birth weight[70]. Moreover, in a sub analysis of the ORIGIN study[71] we demonstrated that low handgrip strength is an important factor associated to an increased risk of cardiovascular mortality in prediabetic and diabetic patients[71]. To explain these results we have proposed that the dramatic increase of overweight and obesity, especially abdominal adiposity, in low and medium income countries[72], is promoting epigenetic adaptations which may alter the leptin/adiponectin (L/A) ratio. This L/A disturbance is in turn the determinant, in populations of low and medium income countries, of their increased vulnerability to the development of IR and an increased risk of cardiovascular events at levels of glycemia that are lower than those used to define DM2[73-76]. Moreover, there are possible regional differences in the risk of developing IR, DM2 and CVD as-

sociated with prediabetes and DM2, as we have recently demonstrated in relation to lung function[77].

PERSPECTIVES TO MODIFY THE CUT-OFF POINTS OF DM RELATED WITH THE RISK OF MACROVASCULAR COMPLICATIONS

The term diagnosis has typically been reserved to characterize or identify individuals with a specific disease. Because the term implies a condition that causes symptoms, tests are often required to confirm the diagnosis. In this order of ideas, when selecting the threshold glucose values, the National Diabetes Data Group[78] acknowledged that "there is no clear division between diabetics and non-diabetics in the FPG concentration or in their response to an oral glucose load" and consequently values were established for each method to identify diabetic patients based on retinopathy and the distribution of plasma glucose population.

Epidemiological studies[10-12] that included an Egyptian population, Pima Indians and the US National Health and Nutrition Examination Survey, all identified retinopathy using fundus photography or direct ophthalmoscopy and by measuring glycemia using FPG, 2-h post-glucose load, and HbA1c, demonstrated that glucose level is a continuous risk factor for retinopathy: the higher levels the higher risk.

Deriving cut points for normal glycemia level from distributions of FPG and 2-h post-glucose load might not be suitable to define cut points for DM because metabolic regulation could varies from population to population. It might be more relevant to base the diagnostic criteria on thresholds for diabetes-specific macrovascular complications, which are probably lower than those for microvascular complications such as retinopathy. Data from the DECODE study[45] which was carried out on behalf of the European Diabetes Epidemiology Group showed that the number of patients diagnosed with DM was one third higher for men and 44% higher for women when using 2-h post-glucose load measurement than when using the FPG, confirming that the 2-h post-glucose load criterion is more accurate than FPG criteria to identify DM. HbA1c is recommended and used in many countries to diagnose DM[12,20]. However the high prevalence of anemia and hemoglobinopathies in under-resourced countries such as ours, together with its high cost, limits its use and from our point of view should not be for now, recommended as a diagnostic test.

The data of the previously mentioned Latin American studies indicate the presence of macrovascular diseases at glycemia levels lower than the internationally established cut points for DM2. These data suggest that the present cut-off points accepted for our population might not be accurate and might have to be reconsidered. Recent studies have shown that the association between dysglycemia and CVD has a considerable increase at levels as low as 100 mg/dL[25,27,45], and therefore, we consider the re-defined cut-points to diagnose DM2 should be around this value. Nevertheless, it is noteworthy that these studies have not been designed for this specific purpose and have not been conducted in Latin America. Thus, as with the risk of microvascular complications, several limitations will be found if we try to re-define the cut-points for DM2 on this basis.

Moreover, as lowering the cut-off points will substantially increase the prevalence of DM2, several public health consequences should be considered before this adjustment. Certainly, diabetic patients require more health care, leading to greater use of resources. In this context, an increased prevalence of DM2 could cause an initial financial challenge of the health systems and household economies in Latin American countries[79]. Nevertheless, indirect economic costs and social consequences attributable to premature mortality and temporary and permanent disability generated as complications of DM should be also considered. Indeed, the direct annual cost associated with diabetes for the year 2000 in Latin America and the Caribbean was estimated as 10721 million US dollars; whereas, the total indirect cost was estimated at almost 54496 million US dollars (mortality, permanent disability and temporary disability accounted for 6%, 92% and 2% of this amount, respectively)[80]. These results suggest a long-term positive cost-effective ratio of an early intervention.

Furthermore, health systems in Latin American countries are based on a model of care with a biomedical curative approach[81], and this has not been favorable in controlling the epidemic of DM2. Thus, health systems should move from an approach of treating DM2 to one of preventing DM2 and its complications. In this way, various socio-medical models are currently being evaluated in Latin-America, such as the ongoing HOPE-4 study in Colombia, in which we are inviting community leaders and non-professional health care workers to form part of the health team to implement new strategies for the detection, prevention and control of non-communicable chronic diseases.

In conclusion, the present challenge for Latin American countries is to conduct population studies in accord with our specific socio-economic conditions, which will permit to establish the cut-point after which lifestyle and/or pharmaceutical interventions must be initiated with the objective of preventing macrovascular complications, associated with hyperglycemia. Further research to assess the economic, public health, and social perspectives is also warranted.

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TOPIC HIGHLIGHT

WJD 5th Anniversary Special Issues (4): Diabetes-related complications

Recent advances on the association of apoptosis in chronic non healing diabetic wound

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Abstract

Generally, wounds are of two categories, such as chronic and acute. Chronic wounds takes time to heal when compared to the acute wounds. Chronic wounds include vasculitis, non healing ulcer, pyoderma gangrenosum, and diseases that cause ischemia. Chronic wounds are rapidly increasing among the elderly population with dysfunctional valves in their lower extremity deep veins, ulcer, neuropathic foot and pressure ulcers. The process of the healing of wounds has several steps with the involvement of immune cells and several other cell types. There are many evidences supporting the hypothesis that apoptosis of immune cells is involved in the wound healing process by ending inflammatory condition. It is also involved in the resolution of various phases of tissue repair. During final steps of wound healing most of the endothelial cells, macrophages and myofibroblasts undergo apoptosis or exit from the wound, leaving a mass that contains few cells and consists mostly of collagen and other extracellular matrix proteins to provide strength to the healing tissue. This review discusses the various phases of wound healing both in the chronic and acute wounds especially during diabetes mellitus and thus support the hypothesis that the oxidative stress, apoptosis, connexins and other molecules involved in the regulation of chronic wound healing in diabetes mellitus and gives proper understanding of the mechanisms controlling apoptosis and tissue repair during diabetes and may eventually develop therapeutic modalities to fasten the healing process in diabetic patients.

Key words: Apoptosis; Diabetes mellitus; Diabetic foot; Chronic wound; Oxidative stress

Core tip: Uncontrolled diabetes mellitus lead to the chronic non healing wound which further can escort to the Ischemia and coronary artery disease. Reports suggested that the involvement of various mechanisms in the development of chronic non healing wound in patients with diabetes mellitus, among which the oxidative stress plays a pivotal role which then leading to the enhanced apoptosis of lymphocytes, may be playing a critical role in the delay of wound healing. Connexins are gap junction protein and their upregulation during diabetes might be leads to improper gap junction formation attributing to the passage of various, apoptotic and inflammatory signals thereby resulting in delayed healing of chronic diabetic ulcers.

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INTRODUCTION

Diabetes mellitus (DM) is a complex, chronic metabolic disorder; affects almost all age group of patients which requires continuous medical care with multifactorial risk reduction strategies beyond glycemic control[1]. Prolonged and uncontrolled DM may leads various complications which is broadly divided into microvascular complications (due to damage to small blood vessels) and macrovascular complications (due to damage to the arteries) affecting several organs, including muscle, skin, heart, brain, and kidneys.

It is reported that patients with DM are increasing rapidly worldwide and it is now recognized that the developing countries like India and China presently face the greatest burden of diabetes. It is the fourth or fifth leading cause of death in most high income countries caused 5.1 million deaths in 2013 and every six seconds a person dies due to diabetes[2]. According to International Diabetes Federation 382 million peoples were diagnosed with diabetes in 2013 which can reach up to 592 million in 2035. Among the countries China and India are having 98.4 and 65.1 million DM patients respectively in 2013 and which could be reach up to 142.7 million in china and 109.0 million in India[2]. Patients with poorly controlled diabetes may be subject to acute complications of diabetes, such as dehydration, poor wound healing, and hyperglycemic hyperosmolar coma.

Patients with DM have 15% higher risk for amputation than the general population due to chronic ulcers. It leads to diabetic neuropathy, which inhibits nociception and the perception of pain[3]. Due to loss of sensation in the feet of DM patients they become unaware of small wounds in the legs and feet, and may consequently fail to prevent infection or repeated injury on time[4]. Further, DM causes immune suppression and damage to small blood vessels, preventing adequate oxygenation of tissue, which can cause chronic wounds[4]. Immune deficiency also takes place in patients with type 2 DM (T2DM) due to the increased apoptosis of lymphocytes[5] and also the increased generation of reactive oxygen species (ROS) in patients with T2DM, might be another factor, which then stimulates downstream apoptotic signalling pathways[6].

In this connection, Desmoulière *et al*[7] reported that the decrease cellularity in wound repair process is achieved by apoptosis of different cell types. It is reported that the reduced rate of apoptosis is correlated with reduced expression of early growth response protein 1 (EGR1) in the 13 d old wound of epidermis of transgenic animal and the EGR1 mediate the proapaptotic signal *via* p53[8] and it clearly vindicated that the induced Egr1 expression plays a critical role in the resolution phase of wound repair by inducing apoptosis in keratinocytes. Further, it is suggested that the Egr1 expression is induced by various proteins among which transforming growth factor beta (TGF-β) is well known[9].

BASIC MECHANISM OF APOPTOSIS

The term "apoptosis" was coined by Kerr *et al*[10] for a morphologically distinct mode of cell death and the other type of cell death is known as necrosis. The key mechanism of apoptosis is endonuclease activation leading to internucleosomal double-stranded chromatin (DNA) fragmentation which occurs in most physiological cell death whereas cell membrane damage takes place in necrosis. Apoptosis is essential, as defects in apoptotic cell death regulation contribute to many diseases including disorders where deregulated cell proliferation occurs (cancer, restenosis) or where cell loss ensues (stroke, heart failure, neurodegeneration, Acquired Immune Deficiency Syndrome)[11]. In wound-healing process apoptosis is responsible for the removal of inflammatory cells and the evolution of granulation tissue into scar tissue[7]. In DM patients delayed wound healing is one of the major problems which are supposed to be takes place due to uncontrolled blood sugar level; it affects apoptosis during the wound healing process[12].

Apoptosis is also known as programmed cell death that may occur in multicellular organisms; leads to characteristic cell changes like blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation[13]. It is a complex process which initiates intracellular apoptotic signalling in response to a stress, which may bring about cell suicide. Cell suicide takes place in four separable but overlapping steps; induction, detection, effectors, and removal[14]. The dying cell remnants are removed by phagocytic cells of the macrophage/monocyte lineage. Interestingly, apoptotic bodies may also be engulfed by cells not specialized in phagocytosis (*e.g.*, vascular smooth muscle cells) (Figure 1)[15].

T2DM is associated with elevated level of oxidative stress, which is one of the most important factors responsible for the development of chronic complications of this disease. Antioxidants like reduced glutathione (GSH), superoxide dismutase (SOD) and catalase protects cells against oxidative damages. In our own publication we have shown that oxidative stress is higher in T2DM patients. In T2DM patients with chronic non healing wound, lymphocyte apoptosis is initiated by the augmentation of reactive oxygen species which leads to the increased expression of proapoptotic proteins like Caspases, FAS, BAX and decreased expression of anti-apoptotic proteins like B-cell lymphoma 2 genes (*Bcl-2*) (Figure 2)[6].

In streptozotocin-induced diabetic rats, the elevated blood sugar level increases cellular apoptosis and the least expression of Bcl-2 protein causes deregulation of the wound healing processes (Tables 1 and 2)[16].

The mechanism of apoptosis has been linked with several proteins but two of them are extensively recognised for their regulation in the pathways (Figure 3)[17]:

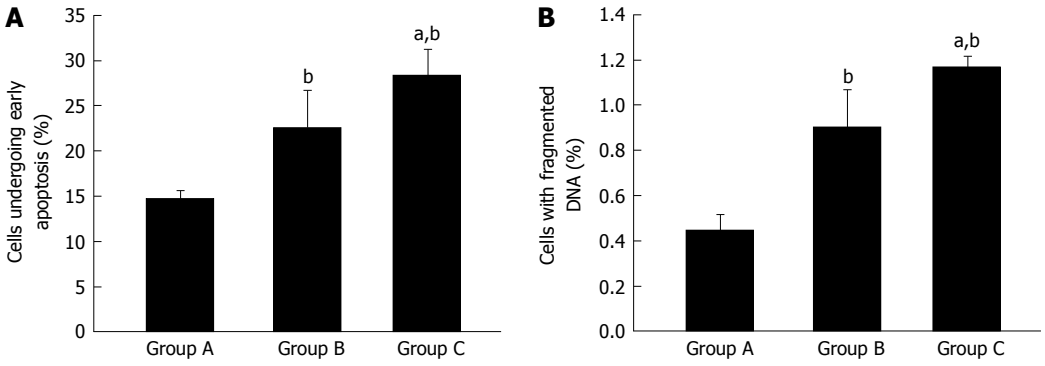

Figure 1 Percentage of apoptotic and dead cells in healthy (Group A), type 2 diabetes mellitus (Group B) and type 2 diabetes mellitus patients with chronic non healing wound (Group C) (A and B). $^{b}P < 0.01$ *vs* healthy; $^{a}P < 0.05$ *vs* uncontrolled diabetes without complication and uncontrolled diabetes with chronic non healing wound. First, second, and third bar in each panel represents healthy, uncontrolled diabetic and uncontrolled diabetic with chronic non healing wound, respectively.

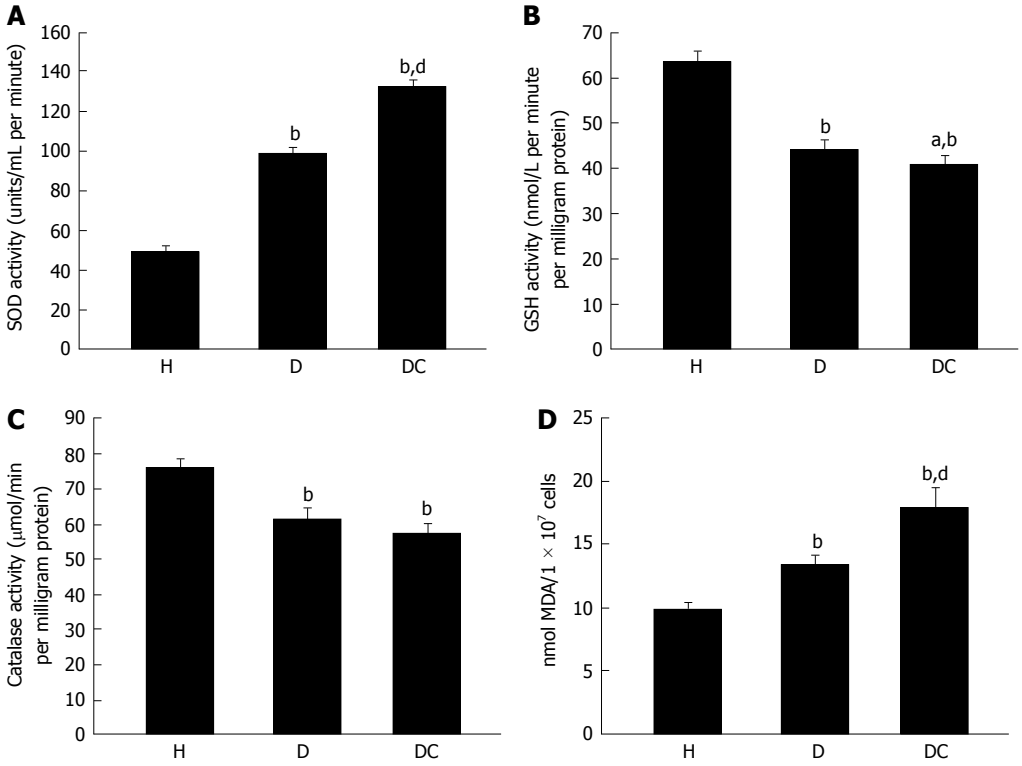

Figure 2 Concentration of superoxide dismutase (A), reduced glutathione (B), catalase (C) and malondialdehyde (D) in healthy (H), type 2 diabetes mellitus (D) and type 2 diabetes mellitus patients with chronic non healing (DC) groups. $^{b}P < 0.01$ *vs* healthy; $^{d}P < 0.01$ and $^{a}P < 0.05$ *vs* uncontrolled diabetes without complication and uncontrolled diabetes with chronic non healing wound. First, second, and third bar in each panel represents healthy, uncontrolled diabetic and uncontrolled diabetic with chronic non healing wound, respectively. SOD: Superoxide dismutase; GSH: Reduced glutathione; MDA: Malondialdehyde.

Table 1 Mean blood glucose level, apoptotic index and DNA fragmentation in control rats (*P* value < 0.01)

	5th day	10th day	20th day	30th day
Control (*n* =10) blood glucose (mg/dL)	75.62 ± 6.41	80.79 ± 11.45	92.05 ± 9.56	90.77 ± 9.7
Apoptotic index (mean ± SD)	1.50 ± 0.60	1.60 ± 0.99	1.64 ± 0.86	1.69 ± 1.12
DNA fragmentation (%) (mean ± SD)	42.25 ± 3.95	44.15 ± 5.61	45.45 ± 5.88	46.58 ± 5.95

(1) targeting mitochondria functionality, or directly transducing the signal *via* adaptor proteins, known as intrinsic pathway; and (2) extrinsic pathway of initiation as identified in several toxin studies is an increase in calcium concentration within a cell caused by drug activity, which can also cause apoptosis *via* calcium binding protease calpain.

In the wound healing process various expression patterns of apoptosis key regulators have been studied

Table 2 Mean blood glucose level, apoptotic index, and DNA fragmentation in rats with diabetes (*P* value < 0.01)

	5th day	10th day	20th day	30th day
With diabetes (n = 10) blood glucose (mg/dL)	467.25 ± 48.2	506.33 ± 35.89	474.99 ± 39.76	488.15 ± 34.36
Apoptotic index (mean ± SD)	3.50 ± 2.60	4.20 ± 2.99	3.60 ± 3.56	3.69 ± 2.75
DNA fragmentation (mean ± SD)	62.80 ± 9.56	74.95 ± 10.45	66.55 ± 8.67	70.48 ± 6.21

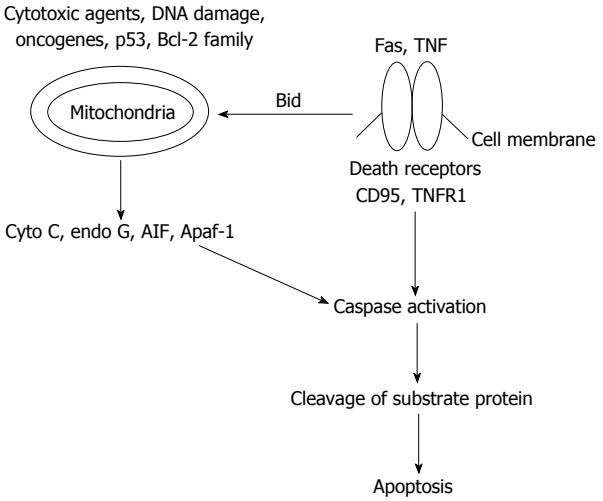

Figure 3 Basic outline of apoptosis mechanism. Bcl-2: B-cell lymphoma 2; TNF: Tumor necrosis factor; AIF: Apoptosis-inducing factor; Apaf-1: Apoptotic protease activating factor-1; TNFR1: Tumor necrosis factor receptor 1.

which shows that the healing in mucosa takes place predominantly through the intrinsic pathway whereas skin healing is predominantly through the extrinsic pathway. The identification of differences in the apoptotic pathways involved in wound healing of various organs may allow the development of therapeutics to improve wound healing[18].

INTRINSIC PATHWAY

The intrinsic signalling pathways involve various arrays of non-receptor-mediated stimuli that produce intracellular signals to work immediately on objects within the cell and are mitochondrial-initiated events. Intrinsic pathway acts both as proapoptotic or antiapoptotic fashion and depends upon the intracellular signals. Negative signals involve the lack of certain growth factors, hormones and cytokines that can escort to collapse of death programs inhibition, thereby triggering apoptosis. Other stimuli that act in encouraging fashion of apoptosis include radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals, *etc*.

Stimulus of apoptotic proteins targeting inner membrane of mitochondria may cause mitochondrial swelling through the formation of mitochondrial permeability transition (MPT) pore, or they may increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out[19]. Formation of MPT is achieved by the group of proteins consist of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi. The release of cytochrome c into the cytoplasm appears to be a crucial step for the activation of caspase. Once cytochrome c is released it binds with Apoptotic protease activating factor-1 and ATP, which then tie up to pro-caspase-9 to create a protein complex known as apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3. Smac/DIABLO and HtrA2/Omi promote apoptosis by inhibiting inhibitors of apoptosis proteins activity[20].

In addition to the release of cytochrome c; apoptosis-inducing factor (AIF), endonuclease G and Caspase Activated DNAse (CAD), discharge from the mitochondria during apoptosis. AIF translocates to the nucleus and causes DNA fragmentation into about 50-300 kb pieces and condensation of peripheral nuclear chromatin[21] whereas Endonuclease G translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments[22]. CAD is subsequently discharged from the mitochondria and translocates to the nucleus where after cleavage by caspase-3, it leads to oligonucleosomal DNA fragmentation and chromatin condensation[23]. The control and regulation of these apoptotic mitochondrial events occur through members of the Bcl-2 family of proteins[24]. Bcl-2 proteins are able to promote or inhibit apoptosis by direct action on MAC/MOMPP. Bax and/or Bak form the pore, while Bcl-2, Bcl-xL or Mcl-1 inhibits its formation.

EXTRINSIC PATHWAY

The extrinsic signaling pathways involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily[25]. Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain"[26]. This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways.

TNF-α signaling is linked to the Fas signaling pathway through the interaction of TNF receptor-associated death domain protein with Fas-associated death domain protein and their activation is critically depends upon the activation of caspase[27]. Once caspase-8 is activated, the execution phase of apoptosis is triggered. The binding of three Fas molecules to a Fas ligand (FasL) homotrimer leads to the subsequent binding of Fas-associated death domain and procaspase-8 which finally triggers a cascade of caspase activation, including caspase-3, leading to cell death[28]. Diabetes-enhanced and prolonged expression of

TNF-α and contributes in the direction of impaired healing[29]. TNF-α is found threefold higher in diabetic mouse wounds than wounds in normal mice[30] and threefold higher found in wound fluid from nonhealing venous leg ulcers than in healing ulcers[31].

EXECUTION PATHWAY OF APOPTOSIS

Execution pathways start from the end point of intrinsic and extrinsic pathways of apoptosis. In this phase execution caspase activates to start organized degradation of cellular organelles. Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10)[23]. Phagocytic uptake of apoptotic cells is the last component of apoptosis. Mice lacking either of these caspases were deficient in skin wound healing and in liver regeneration[32].

Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the characteristic feature of cell death which can be measured by fluorescent activated cell sorter using annexin V tagged with fluorescent molecule[5].

DIABETIC WOUND HEALING AND APOPTOSIS

Usually wound healing process can be split into 4 temporarily and spatially overlapping phases: coagulation, inflammation, tissue formation (proliferative phase) and tissue remodelling or scar formation phase.

COAGULATION PHASE

Coagulation phase takes place immediately after injury to stop excessive blood flow from wound and provides provisional protection for the wounded area. Hemostatic reaction started with the adherence of platelets to damaged blood vessels giving rise to a blood-clotting cascade. To facilitate aggregation platelates express sticky glycoproteins on their cell membrane[33]. Platelets also released cytokines and growth factors which are a potent chemotactic agent; stimulates the deposition of extracellular membrane to the wound site[34]. In addition, platelets release proinflammatory factors like serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane, and histamine to dilate blood vessel and increase cell proliferation and migration to the wound area[35].

INFLAMMATORY PHASE

Inflammatory phase starts with the release of platelet-derived growth factor and TGF-A1 and TGF-2 from platelet which attract inflammatory cells, such as leukocytes, neutrophils, and macrophages[36]. Leukocytes release ROS that are antimicrobial and proteases that clear the wound of foreign bodies and bacteria. T lymphocytes playing central role in the wound healing[37] and its increased apoptosis leading to delayed wound healing in diabetic patients[17]. Neutrophils are important in wound healing as they serve to control infection by eliminating microorganisms. With the control of infections neutrophils also release harmful enzymes which damage healthy tissues surrounding the wound site. To prevent further inflammation neutrophils are engulfed by macrophages during the process of apoptosis[38]. Macrophages are the key scavengers for resolving inflammation and facilitating tissue regrowth[39]. These findings show that apoptosis of immune cells could be the major key to end inflammation and initiate healing[40].

Diabetes impaired wound healing by reducing macrophage number and activation which results in the reduced lymphatic vessel formation[41]. The anti proliferative protein p53 involved in apoptosis of inflammatory cells during the healing process and its expression during the healing of cutaneous wounds in swine has been reported by Antoniades *et al*[42].

PROLIFERATIVE PHASE

Proliferative phase of repair begins with the settling down of inflammatory phase and formation of granulation tissue. Granulation tissue formation takes place by growth factors which are released by basal keratinocytes, remaining inflammatory cells and migrating epidermal and dermal cells to support the epithelialization process of wound healing[36]. Diabetes mellitus affects re-epithelialization by affecting multiple proteins and genes including angiopoietin-4[43]. ANGPTL4 shows a potential effect on lipid homeostasis, glucose metabolism, re-epithelialization, inflammation, and potential effect on energy homoeostasis, which is required for wound healing. In corneal wound healing; apoptosis of stromal keratinocyte is well characterised. It triggers subsequent cellular processes that include bone marrow-derived cell infiltration, proliferation, and migration of residual keratinocyte cells and in some circumstances, generation of myofibroblast cells[44].

Diabetes mellitus affects signalling intermediates responsible for coordinating/regulating wound healing angiogenesis and vasculogenesis[45]. Due to the deficiencies in either endothelial progenitor cell or peripheral tissue homing and engraftment of bone marrow, diabetic patients are prone to the development of chronic wounds[46].

TISSUE REMODELING

Tissue remodeling is the process of reformation or restoration of existing tissues. Restoration of a normal blood supply offers an encouraging microenvironment for epidermal and dermal cell migration and proliferation. Fibroblasts proliferate within the wound and synthesize extra-cellular matrix (ECM) forming granulation tissue perfused with newly formed blood vessels.

Wound contraction and matrix remodeling occurs

after the substitution of ECM from collagen Ⅲ, fibrin, fibronectin, and hyaluronic acid[36]. Collagen homeostasis is aberrant in the wound of uncontrolled DM patients who suppose to be mediated by Hsp47; leading to the dysfunction of fibroblast cells. Such impairments could contribute to delayed wound healing[47]. With wound maturation, different cell populations need to be eliminated. Apoptosis of fibroblastic cells occurs, leading to the formation of a relatively acellular scar tissue whose tensile strength is equivalent with unwounded skin. Early studies suggest that endothelial cells undergo apoptosis followed by the removal of myofibroblasts[48].

The passage of various apoptotic and inflammatory signals *via* gap junctions play an important role in tissue remodelling during diabetic wound healing. Connexins (Cx), the gap junction proteins, form channels between two adjacent cells and their expression is highly regulated after wound formation at the transcriptional, translational and post translational levels[49]. In diabetic wounds significant increase in the levels of Cx26, Cx30.3, Cx31, Cx31.1, and Cx43 were observed as compared to non-diabetic wounds[50]. An up regulated connexin expression might lead to the improper gap junction formation attributing to the passage of various, apoptotic and inflammatory signals thereby resulting in delayed healing of chronic diabetic ulcers.

CONCLUSION

Diabetes mellitus delayed normal wound healing by various ways like narrowing of the blood vessels due to arteriosclerosis or leading decreased blood flow and oxygen to a wound, loss of sensation in feet and lowering down the efficiency of the immune system. DM is leading various complications like macroangiopathy and microangiopathy among which Chronic wounds such as venous ulcers are rapidly increasing. In chronic non healing DM patients various cytokines and chemokines are interacting together to lead various complications, *e.g.*, strong positive association between interleukin-7 and monocyte chemoattractant protein 1 may be a possible cause of developing coronary artery disease in these patients[51]. Dysregulation of apoptosis in response to hyperglycemia is universal, leading to impaired wound healing along with the involvement of other target organs. Contrary to the accepted view that diabetic foot is caused by neuropathy and peripheral vascular disease, it now appears that dysregulated apoptosis is emerging as a major cause of the diabetic foot wound. Recent advances in management of DM and understanding of the molecular and cellular components of apoptosis involved during the wound healing phases may enable personalized diagnosis and therapy tailored to a particular patient's needs and therefore lead to better therapeutic outcomes.

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REVIEW

Biomarkers in diabetic nephropathy: Present and future

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Abstract

Diabetic nephropathy (DN) is the leading cause of end stage renal disease in the Western world. Microalbuminuria (MA) is the earliest and most commonly used clinical index of DN and is independently associated with cardiovascular risk in diabetic patients. Although MA remains an essential tool for risk stratification and monitoring disease progression in DN, a number of factors have called into question its predictive power. Originally thought to be predictive of future overt DN in 80% of patients, we now know that only around 30% of microalbuminuric patients progress to overt nephropathy after 10 years of follow up. In addition, advanced structural alterations in the glomerular basement membrane may already have occurred by the time MA is clinically detectable.Evidence in recent years suggests that a significant proportion of patients with MA can revert to normoalbuminuria and the concept of nonalbuminuric DN is well-documented, reflecting the fact that patients with diabetes can demonstrate a reduction in glomerular filtration rate without progressing from normo-to MA. There is an unmet clinical need to identify biomarkers with potential for earlier diagnosis and risk stratification in DN and recent developments in this field will be the focus of this review article.

Key words: Diabetes; Nephropathy; Microalbuminuria; Proteinuria; Biomarkers

Core tip: Microalbuminuria (MA) is the earliest and most commonly used clinical index of diabetic nephropathy (DN), however its sensitivity and specificity for early disease detection are limited. Not all patients with MA progress to overt DN, nonalbuminuric DN is common and risk associated with MA is elevated even at levels below currently accepted diagnostic thresholds. There is therefore a need for alternative biomarkers allowing early identification of "at risk" individuals. This review focusses on biomarkers of glomerular and tubular dysfunction, oxidative stress and inflammation that have attracted interest. In addition we review more novel strategies including proteomic, metabolomic and genomic approaches.

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INTRODUCTION

The global incidence of type 2 diabetes continues to rise due to the increase in obesity and the aging population. In 2000 the prevalence of diabetes was estimated to be 171 million (2.8%) worldwide. It is projected that by 2030, 366 million (4.4%) people worldwide will have diabetes[1,2]. Diabetic nephropathy (DN), defined as albuminuria (albumin excretion rate > 300 mg/24 h) and declining renal function in a patient with known diabetes in the absence of urinary tract infection or any other renal disease[3], is the leading cause of end stage renal disease

in the Western world. In the 1960s the development of assays for detection of microalbuminuria (MA) revolutionised diabetes management[4]. MA, defined as urinary albumin excretion rate (UAE) 30-300 mg/d, is the earliest and most commonly used clinical index of DN. MA is independently associated with cardiovascular risk in diabetic patients[5-8], due in part to its role as an indicator of widespread microvascular disease and of underlying renal disease, and studies have since indicated that a reduction of UAE in type 2 diabetic patients reflects renal and cardiovascular risk reduction[9]. Consequently, UAE has become a key therapeutic target in the management of patients with diabetes. Evidence from the Diabetes Control and Complications Trial and United Kingdom Prospective Diabetes Study Group proved that tight glycaemic and blood pressure control can reduce risk of microvascular complications of diabetes including DN[10-12] for patients with type 1 or type 2 diabetes respectively and this strategy forms the basis of current management guidelines for microalbuminuric patients.

Although UAE remains an essential tool for risk stratification and monitoring disease progression a number of factors have called into question its sensitivity and specificity. The presence of MA was originally thought to be predictive of future overt DN in 80% of patients. However more recent evidence suggests that only around 30% of microalbuminuric patients progress to overt nephropathy after 10 years of follow up[13]. It has also been shown that advanced structural alterations in the glomerular basement membrane may already have occurred by the time MA becomes clinically evident[14,15]. In addition, there is evidence that a significant proportion of patients with MA can revert to normoalbuminuria[16] and the concept of nonalbuminuric DN is well-documented, reflecting the fact that patients with diabetes can demonstrate a reduction in glomerular filtration rate without progressing from normo-to MA[14,17]. Taken together, these results suggest that MA is perhaps more a diagnostic marker than a tool to predict DN. Therefore, there is a need to identify and investigate alternative biomarkers for the earlier prediction of DN and these are subject to this review.

GLOMERULAR FILTRATION

Glomerular filtration rate (GFR) is the best marker of renal excretory function. The current gold standard methods for determining GFR in the research setting are inulin and ^{51}Cr-EDTA plasma clearance. The time-consuming and labour intensive nature of these techniques, as well as requirement of experienced personnel, however, mean that they are not routinely available in clinical practice. Here the most commonly used index for assessment of GFR is serum creatinine, although its sensitivity is poor in the early stages of renal impairment, as by the time an increase in serum level is detectable, a significant decline in GFR has already taken place[18]. Formulae using serum creatinine to estimate GFR (eGFR) such as the Modification of Diet in Renal Disease equation are not reliable at GRF > 60 mL/min per 1.73 m^2. The recently developed Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula appears to be more accurate in patients whose GFR is > 90 mL/min per 1.73 m^2[19-21] however a marked underestimation of GFR in diabetic patients continues to be evident using this equation when compared to its performance in healthy individuals[22]. The current Kidney Disease Improving Global Outcomes guidelines staging system classifies chronic kidney disease stages 1 and 2 using GFR cut-offs of > 90 mL/min and 60-89 mL/min respectively[23]. Routine clinical tests therefore do not measure this degree of GFR decline accurately, meaning that this potentially critical early stage of renal dysfunction remains undetected[24].

Cystatin C (CysC) based assays in estimating GFR for clinical trials in DN offer an alternative approach due to the complexity and time-consuming nature of other reference test methods. This 13.3 kDa plasma protein is freely filtered through the glomerulus and reabsorbed and catabolised by tubular cells to such a degree that it does not return to the blood in an intact form[25]. Numerous studies have validated CysC as a marker of renal function[26-28]. Its levels are well correlated with GFR and unlike serum creatinine, are unaffected by muscle mass. In addition CysC levels not only correlate with progression of nephropathy, but also show a more sensitive marker of early DN when eGFR remains > 60mL/min[29-31]. These benefits should, however, be taken into consideration alongside the higher cost of the immunoassay and the greater intraindividual variability[28] compared to serum creatinine Formulae for estimating GFR including both creatinine and CysC have been proposed but to date have not been proven to enhance precision in identifying and monitoring early stages of GFR decline in diabetes[32].

MARKERS OF GLOMERULAR DYSFUNCTION

Glomerular damage increases permeability to plasma proteins resulting in their excretion in the urine. In addition, abnormalities of extracellular matrix synthesis and degradation in kidney disease can lead to increased urinary excretion of matrix proteins, reflecting glomerular injury. Although albumin excretion remains the current gold standard marker of glomerular damage in the clinical setting, a number of other proteins have been proposed as useful indicators of early glomerular damage.

Transferrin is a plasma protein with a slightly greater molecular weight (76.5 kDa) than albumin[33]. It is also less ionic than glycosylated albumin and thus less easily repelled by glomerular basement membrane polyanion[34]. Elevated urinary transferrin excretion has been demonstrated in patients with diabetes compared with healthy controls, even in in the absence of albuminuria[35]. Transferrinuria has been shown to correlate with UAE and to increase in parallel with it[36]. In a 24 mo follow up study it has been demonstrated that increased urinary transferrin

excretion predicted development of MA in a cohort of normoalbuminuric type 2 diabetic patients independent of age, diabetes duration, blood pressure, HbA1c and baseline lipid levels[33]. Elsewhere it has also been shown that transferrinuria predicted development of MA at 5 years follow up[36]. Transferrin has also been proposed as a mediator of tubular toxicity, as its reabsorption results in release of reactive iron in proximal tubular cells promoting formation of hydroxyl radicals[37,38]. Studies have reported correlations between urinary transferrin excretion and other microvascular diabetic complications such as retinopathy[38]. Taken together, the above data suggest that transferrinuria may serve as a sensitive indicator of early proteinuria and increased vascular permeability.

Accumulation and altered distribution of basement membrane components is one of the structural hallmarks of DN and these changes precede the development of MA[39]. Type Ⅳ collagen is a normal constituent of mesangial matrix as well as tubular and glomerular basement membranes, with molecular weight of 540 kDa. Both serum and urine levels have been shown to be elevated in patients with diabetes[40]. Urinary type Ⅳ collagen excretion has been shown to correlate closely with degree of UAE, as well as diabetes duration, blood pressure and serum creatinine[41,42]. Significantly higher excretion of type Ⅳ collagen has been found even in normoalbuminuric diabetic patients as well as patients with impaired glucose tolerance, suggesting that this may serve as an early indicator of DN, preceding the onset of MA[42,43]. In addition, type Ⅳ collagen excretion has been found to decrease with improved glycaemic control, suggesting that this marker is also reversible in early disease[44]. Type Ⅳ collagen may also play a role in differentiating DN from other non-diabetic kidney diseases, as the ratio of type IV collagen to albumin has been found to be significantly higher in DN in comparison to other glomerulopathies[40].

Ceruloplasmin is a 132 kDa acute phase protein with well characterised functions in the metabolism of copper and iron[36]. It has been suggested that ceruloplasmin may leak through glomerular capillary walls in DN and evidence confirms increased excretion in both impaired glucose tolerance and diabetes compared with healthy controls[36,45]. Increased urinary ceruloplasmin excretion has also been demonstrated in normoalbuminuric patients with diabetes[45]. In addition, urinary ceruloplasmin excretion appears to parallel UAE[31,46]. In a 5 year follow up study, it was demonstrated that increased urinary ceruloplasmin excretion predicted development of MA in normoalbuminuric type 2 diabetic patients[36]. Improved glycaemic control appears to reverse this increase[46].

Fibronectin is a high molecular weight (440 kDa) plasma glycoprotein mainly produced by endothelial cells and fibroblasts which plays a role in cell adhesion to vascular endothelium[35]. Fibronectin biosynthesis is increased in patients with diabetes and studies have suggested that plasma levels correlate with retinopathy and MA[47]. Increased urinary levels of fibronectin have been found in type 2 diabetic patients in comparison with healthy controls, as well as in subjects with MA compared to normoalbuminuric subjects[47]. However, there is only a weak positive correlation between plasma fibronectin and urinary albumin levels perhaps limiting its potential usefulness as an early marker of DN[47], and there is no published evidence comparing urinary fibronectin with UAE in terms of predictive value for diabetic nephropathy.

MARKERS OF TUBULAR DYSFUNCTION

Plasma proteins of low molecular weight are excreted in increased quantities in the urine due to deficient tubular reabsorption or increased secretion by tubular epithelial cells. Similarly, urinary enzymes are thought to be sensitive markers of tubular damage as they are not filtered at the glomerulus due to their high molecular weight[31,36].

Neutrophil Gelatinase-Associated Lipocalin (NGAL) is a small molecule of 25 kDa belonging to the lipocalin superfamily. These proteins play a role in binding and transporting small hydrophobic molecules, apoptosis and immune regulation. NGAL is stored mainly in the specific granules of neutrophils and also expressed at low levels in several other human tissues[48,49]. NGAL shows significant promise in the diagnostic and clinical setting as a marker of acute kidney injury[48] and is thought to also play a renoprotective role as a mediator of tubular cell proliferation[49]. Studies have confirmed an association between NGAL and obesity, insulin resistance and hyperglycaemia in human subjects[49]. Urinary NGAL concentration has been found to be increased in diabetic subjects compared with healthy controls[50] and to correlate negatively with eGFR, and positively with CysC, serum creatinine and urea in patients with type 2 diabetes[48]. Significant increases in urinary NGAL concentration have been demonstrated from normo- to micro- to macroalbuminuric groups of patients with type 1 diabetes[51]. Similar results have been published in a study of type 2 diabetic patients[52]. Urinary NGAL correlates positively with glomerular hyperfiltration early in the clinical course of diabetes[53] and higher values have been found to be associated with enhanced decline in eGFR in type 2 diabetes patients with proteinuria, although this correlation was no longer statistically significant after adjustment for factors including systolic blood pressure, HbA1c and diabetes duration[53]. However, other prospective studies have not confirmed these associations[54,55] and further investigation of the role of urinary NGAL in DN is required.

Kidney injury molecule 1 (KIM1) has been shown to be a marker of tubular damage in various chronic kidney diseases[56,57]. This type 1 cell membrane glycoprotein is expressed on the apical membrane of proximal tubule cells and is involved in the phagocytosis of damaged cells in the proximal tubules[52]. Expression is undetectable in normal healthy kidneys but mRNA and protein are markedly upregulated in acute kidney injury[58]. In a cross-sectional study urinary KIM1 excretion has been

found to be increased in diabetic patients compared to healthy controls. A weak but significant increase of urinary KIM1 concentration was noted with increasing degree of UAE[50]. Increased urinary KIM1 excretion has also been shown in type 2 diabetics with glomerular hyperfiltration[52]. In a 3 year prospective interventional study, high baseline levels of urinary KIM1 were found to be associated with faster decline in GFR in type 1 diabetes with DN; an association no longer significant after adjustment for traditional risk markers including blood pressure and glycaemic control[58]. Similar findings have been described in type 2 diabetes populations[55]. Studies have shown that treatment with renin angiotensin system (RAAS) blocking agents reduced urinary KIM1 excretion in parallel to reductions in blood pressure and UAE[59]. In addition, low baseline urinary KIM1 excretion is strongly associated with regression of MA during a 2 year follow up period, independent of clinical characteristics[57]. This supports the hypothesis that KIM1 is a good marker of active tubular damage, rather than pre-existing scarring[58].

N-acetyl-b-d-glucosaminidase (NAG) is a lysosomal enzyme which is predominantly located in the renal tubules. It cannot be filtered from blood through an intact glomerular membrane due to its high molecular weight (140 kDa), thus its activity detected in urine reflects tubular dysfunction. Urinary NAG activity is increased in a variety of tubulointerstitial diseases. It is elevated in populations with diabetes compared to controls, even in normoalbuminuric patients[33,53]. It correlates with the degree of UAE and excretion of transferrin and creatinine[60-62]. Although no significant association has been found between urinary NAG and glomerular hyperfiltration[52], prospective follow up studies have shown that higher levels of NAG at baseline are predictive of subsequent DN[63]. In addition, lower baseline NAG levels are significantly associated with regression of MA at follow up[57]. Finally, significant increases in NAG excretion have been reported in type 2 diabetic patients with both micro- and macrovascular complications[63-65] and in fact NAG levels have been attributed comparable diagnostic value to UAE in this regard[65].

Liver-type fatty acid binding protein (L-FABP) is a low molecular weight (15 kDa) intracellular carrier protein that is expressed in the proximal tubule and liver[66,67]. It is produced in response to tubulointerstitial compromise, and thus has potential as a marker of structural and functional renal tubular damage[67]. In a cross sectional study of patients with type 1 diabetes and varying degrees of UAE, urinary L-FABP levels were significantly higher compared to healthy controls. The levels increased with increasing degree of albumin excretion. Intervention with Lisinopril was associated with significant reductions in UAE and urinary L-FABP excretion in those with diabetes[68]. However, there is no correlation between L-FABP and rate of change of eGFR in patients with type 2 diabetes[54]; therefore further studies are needed to elicudate its value as a predictive marker for DN.

Low molecular weight proteins are freely filtered at the glomerulus and some have been used as markers of tubular damage in various renal diseases[36]. β2-microglobulin (β2MG) is a 11.8 kDa protein produced by cells expressing major histocompatibility class 1. Urinary β2MG excretion is known to be elevated in patients with reduced GFR and some evidence links β2MG with tubular injury[69]. β2MG has also been associated with macrovascular complications in type 2 diabetes[63]. However, its diagnostic utility is limited by its poor stability at acidic pH[70]. The stable microprotein α-1-microglobulin (A1M) may offer an alternative means of evaluating tubular function. This 26 kDa glycoprotein is freely filtered at the glomerulus and almost completely reabsorbed in the proximal tubules, thus even minor degrees of proximal tubular dysfunction lead to increased urinary A1M excretion[71,72]. Urinary A1M excretion has been shown to be greater in patients with type 2 diabetes compared to healthy controls[33,42]. A1M levels have also been found to correlate with diabetes duration and degree of diabetes control[63,71]. There is evidence that urinary A1M excretion significantly increases with degree of MA in type 2 diabetes[71-73]. However, Hong *et al*[72] found in a cross-sectional study that although UAE and A1M were directly related, in some patients one could be present in the absence of the other, suggesting that urinary A1M (as a measure of tubular function) may be complementary to MA (as a measure of glomerular function) in assessment of early DN. Retinol binding protein (RBP) is another low molecular weight protein (21 kDa) which is freely filtered at the glomerulus and almost completely reabsorbed in the proximal tubule; as such its presence in the urine is indicative of even very minor degrees of tubular dysfunction[33]. Increased urinary RBP excretion has been described in diabetic patients compared to controls, even in patients with normal UAE[16,70,73]. RBP levels have also been found to correlate with both micro- and macrovascular complications in type 2 diabetic patients[64,74]. RBP, therefore, may also have a complementary role in early detection of DN together with biomarkers of glomerular damage such as UAE or transferrin. Immunoglobulin free light chains (FLCs) kappa and lambda undergo similar glomerular filtration and near complete tubular reabsorption[36]; consequently their presence in the urine can also be indicative of proximal tubular dysfunction[75]. Abnormal urinary FLCs/creatinine ratio in type 2 diabetes patients, both with normal and elevated UAE, and FLC excretion appears to be increased before overt renal disease occurs[76]. However, as yet there is little further published evidence regarding use of FLCs as a predictive tool for early detection of DN.

MARKERS OF OXIDATIVE STRESS AND INFLAMMATION

Oxidative stress is thought to be one of the key mediators of vascular complications of diabetes. Generation of reactive oxygen species (ROS) as a result of hyperglycaemia contributes to development of diabetes com-

plications through sorbitol accumulation, formation of advanced glycation end products (AGE) and activation of protein kinase C[77,78].

8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) is a product of oxidative DNA damage resulting from specific enzymatic cleavage after ROS-induced 8-hydroxylation of the guanine base in nuclear and mitochondrial DNA[78]. Since it is excreted into urine without being further metabolised its urinary concentration serves as an index of oxidative stress[79]. Increased concentrations of 8-OHdG have been described in both urine and mononuclear cells of diabetic patients[80], and urinary excretion appears to correlate closely with the severity of DN and retinopathy as well as HbA1c[81]. In a prospective longitudinal study of 532 Japanese diabetic patients, urinary 8-OHdG excretion at baseline was associated with later development of DN after 5 years of follow up[81], indicating its potential as a clinical predictive marker.

AGE have been associated with the pathogenesis of diabetes complications[82]. AGE-modified proteins generally undergo glomerular filtration and subsequent catabolism at the proximal tubule, thus it seems intuitive that the presence of AGE-modified protein fragments in urine may also herald early tubular dysfunction**.** Pentosidine is one of the major molecular structural components of AGEs and acts as a marker of their formation and accumulation[83]. Urinary excretion of Pentosidine has been shown to be higher in patients with diabetes compared to healthy controls[84]. Increased urinary and plasma Pentosidine levels have been demonstrated in patients with DN[85]. More recently its potential as a marker of microvascular complications of diabetes has been shown with associations between serum Pentosidine levels and diabetic retinopathy, hypertension and hyperlipidaemia in addition to DN[86]. Although initially no correlation between Pentosidine levels and UAE were reported[84], recent publications have challenged this finding; one study reported significantly increased serum Pentosidine levels in diabetes patients with MA compared to normoalbuminuric controls[87] and another study found increased median urinary Pentosidine excretion in diabetes patients with macroalbuminuria compared to controls[62]. In addition, this study demonstrated that baseline urinary Pentosidine excretion predicted later macroalbuminuria, with risk increasing almost 7-fold for every 50% increase in urinary Pentosidine[62].

Evidence is accumulating that immune and inflammatory mechanisms also play a role in the pathogenesis of DN[88], as cause rather than consequence of disease[89]. Individuals who progress to DN appear to display features of low grade inflammation for years before clinically detectable disease[90,91]. As a result, cytokines and other components involved in the process of inflammation and endothelial damage have attracted attention as potential markers of DN.

Orosomucoid, or α-1-acid glycoprotein (AGA) is a single chain polypeptide produced mainly by the liver. It is released in response to inflammation under the stimulation of cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α)[92]. AGA levels have been found to be associated with ischaemic heart disease, lung cancers and diabetes[92,93]. It has been suggested that high AGA levels may predict the development of type 2 diabetes[94]. In a cross sectional study of outpatients with type 2 diabetes and no known cardiovascular disease, serum AGA levels were found to correlate significantly with UAE[95]. In addition, proteomic work has identified urinary AGA as an independent risk factor for DN[96,97]. Urinary AGA excretion appears to increase in parallel with UAE and data indicate that urinary AGA is elevated in the early stages of DN[95]. The potential predictive value of urinary AGA in DN has been shown[98] but further work is needed to determine whether AGA could be used as a biomarker of disease development and treatment response.

TNF-α and IL-6 are two major pro-inflammatory cytokines that stimulate the acute phase response by triggering production of other proteins such as CRP and AGA[89,93]. Patients with DN have higher serum and urinary concentrations of TNF-α than healthy controls or normoalbuminuric subjects[99,100]. Urinary TNF-α excretion also appears to be increased in diabetes patients with micro- or macroalbuminuria compared to normoalbuminuric patients[100,101], with one study reporting an increase of 90% between normo- and microalbuminuric patients[100]. Urinary TNF-α excretion has also been shown to correlate with NAG excretion, a marker of severity of tubular damage[99]. TNF-α mediates its effects *via* two distinct receptors, TNF receptor 1 (TNFR1) and TNFR2, which are both membrane bound and also can be found in serum in soluble form[102]. Serum levels of both these receptors have been shown to correlate with GFR in diabetic patients independently of albuminuria status[102]. More recent data suggest that serum concentrations of TNFR1 and TNFR2 have potential as predictors of progressive renal disease in diabetes[103,104]. Patients with TNFR levels in the highest quartile show significantly elevated cumulative incidence of reaching stage 3-5 CKD over 12 years of follow up compared with those in the lower quartiles. This has been shown in both type 1 and type 2 diabetes, in the presence or absence of proteinuria[103,104].

Serum IL-6 has been shown to be elevated in patients with diabetes compared to control subjects, as well as between normo-, macroalbuminuric and overtly proteinuric patient groups[105,106]. In addition, IL-6 has been linked to glomerular basement membrane thickening[106]. Furthermore, association has been demonstrated between circulating levels of both TNF-α and IL-6 and micro- and macrovascular complications of diabetes[107].

Vascular endothelial growth factor (VEGF) is a potent cytokine that induces angiogenesis and increases endothelial permeability[108]. It adversely affects the glomerular filtration barrier by enhancing its permeability to macromolecules and exacerbating proteinuria[109]. Urinary VEGF excretion appears to be elevated in patients with

Table 1 Summary of biomarkers with potential utility in diagnosis of diabetic nephropathy

Biomarker	Serum/plasma or urine	Type of marker	Status in DN	Potential for additional information beyond UAE	Ref.
Transferrin	Urine	Glomerular	Elevated	Predicts MA	[30-35]
Type Ⅳ collagen	Urine	Glomerular	Elevated	Rises in parallel with UAE, even in nonalbuminuric stage	[36-41]
Ceruloplasmin	Urine	Glomerular	Elevated	Predicts MA	[33,42-44]
Fibronectin	Plasma/urine	Glomerular	Both elevated	No	[32,45]
NGAL	Urine	Tubular	Elevated	Marker of glomerular hyperfiltration	[46-53]
KIM1	Urine	Tubular	Elevated	Marker of glomerular hyperfiltration	[49,50,53-57]
NAG	Urine	Tubular	Elevated	Comparable to UAE	[30,58-64]
L-FABP	Urine	Tubular	Elevated	No	[52,65-66]
A1M	Urine	Tubular	Elevated	No	[30,39,63,69-74]
RBP	Urine	Tubular	Elevated	No	[17,30,69,72-75]
FLCs	Urine	Tubular	Elevated	No	[17,63,69,72-75]
8-OHdG	Urine	Oxidative stress	Elevated	Predicts DN but value in comparison to MA remains unclear	[77-80]
Pentosidine	Urine/serum	Oxidative stress	Both elevated	No	[61,81-86]
AGA	Urine	Oxidative stress	Elevated	Urinary excretion predicts MA	[91-97]
TNF-α	Urine/serum	Inflammatory	Both elevated	No	[88,92,98-100]
TNFR 1/2	Serum	Inflammatory	Elevated	Predictive of onset of stage 3-5 CKD independent of albuminuria status	[99-101]
IL-6	Urine/serum	Inflammatory	Serum levels elevated	No	[99,101-103]
VEGF	Urine/serum	Inflammatory	Urinary levels elevated	No	[104-108]

DN: Diabetic nephropathy; NGAL: Neutrophil gelatinase associated lipocalin; KIM1: Kidney injury molecule 1; NAG: N-acetyl-b-d-glucosaminidase; AIM: α-1-microglobulin; L-FABP: Liver type fatty acid binding protein; RBP: Retinol binding protein; FLCs: Free light chains; 8-OHdG: 8-oxo-7,8-dihydro-2′-deoxyguanosine; AGA: α-1-acid glycoprotein; TNF-α: Tumour necrosis factor α; TNFR 1/2: Tumour necrosis factor α receptors 1 and 2; IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; CKD: Chronic kidney disease; UAE: Urinary albumin excretion; MA: Microalbuminuria.

diabetes, even at the normoalbuminuric stage[109,110]. A significant increased urinary excretion of VEGF in micro- and macroalbuminuric type 1 diabetic patients has been demonstrated[110]. Work in type 2 diabetes demonstrated that urinary VEGF concentration increases with DN stage. This has not been demonstrated in plasma[109]. However, baseline serum VEGF level did appear to be predictive of subsequent DN in a follow up study of children with type 1 diabetes[111]. In addition, both serum and urinary VEGF levels have been shown to be elevated in patients with diabetic retinopathy, although the sensitivity of urinary detection was poor[112]. Taken together, these findings led to the proposal that plasma VEGF is a reliable marker of generalised vascular dysfunction and retinopathy, whereas urinary concentration may serve as a sensitive predictor of risk of subsequent MA[109] (Table 1).

GENETIC FACTORS

In 1989 Seaquist *et al*[113] demonstrated strong familial clustering of DN, triggering a search for associated genetic variants. However, identifying gene variants that predispose to DN is complex as susceptibility is likely to be determined by a large number of common allelic variants, each of which may confer a modest increase in relative risk. In addition, overall risk of developing DN is a result of a combination of both genetic and environmental influences. Advances in genotyping technology have led to use of genome wide association scans (GWAS) for studying disease susceptibility across the entire genome. In relation to DN the creation of groups such as Family Investigation of Nephropathy and Diabetes (FIND) and Genetics of Kidneys in Diabetes (GoKinD) have facilitated such research.

The FIND group is a large multicentre consortium making use of family based linkage analyses in multiethnic groups to identify genes with significance in type 2 DN[114]. Results of the group's preliminary genome scan observed evidence linking chromosome loci 7q21.3, 10p15, 14q23.1 and 18q22.3 with DN[115]. Further publications by the group have shown a significant contribution of chromosomes 1q43, 8q13.3 and 18q23.3 to eGFR phenotype[116], and suggested contribution of chromosomes 3p, 7q, 16q and 22q to UAE status in African-American and European-American populations[117].

GoKinD group have accumulated a collection of DNA for genetic association studies of DN in the context of type 1 diabetes[118]. This group have identified genetic associations for DN susceptibility at candidate loci near the *FRMD3* and *CARS* genes[119]. In addition, variants in the *ELMO1* gene on chromosome 7p have previously been linked with DN in Japanese and African-American populations with type 2 diabetes[120]. GWAS data from the GoKinD collection confirmed this association in a Caucasian population[121].

A genome wide linkage scan in Diabetes Heart Study families detected significant evidence for linkage with eGFR on chromosomes 2p16, 7q21 and 13q13. Evidence for linkage to UAE however was far weaker[122]. In addition, genome wide DNA methylation analysis in a case control study of 192 Irish patients with type 1 diabetes identified 19 prospective CpG sites associated with risk of DN[123]. In 2012 the Genetics of Nephropathy: an International Effort consortium undertook a meta-analysis

of GWAS of DN in type 1 diabetes. They identified signals in an intron in the *AFF3* gene on chromosome 15 and linked this to DN mechanistically by providing evidence that AFF3 expression is linked to transforming growth factor beta-driven fibrosis in cultured epithelial cells[124,125]. Although this locus technically did not replicate, the potential for misclassification through identifying cases using clinical rather than histological criteria may have led to reduced statistical power[124].

PROTEOMICS

Proteomics is the study of the proteome, reflecting the protein content of the genome, and is defined as "the knowledge of the structure, function and expression of all proteins in the biochemical or biological context of organisms"[126]. These methods have attracted attention in recent years as a potentially important tool for early, pre-clinical disease detection as they allow simultaneous examination of the patterns of multiple urinary and plasma proteins. In view of the complex pathogenesis of type 2 diabetes, it is perhaps simplistic to expect that a single biomarker will provide sufficient sensitivity and specificity for disease prediction, detection and treatment monitoring, and therefore such multimarker approaches are appealing. Both urinary and plasma proteome analysis have identified a number of biomarkers which are significantly associated with DN, such as specific collagen fragments[127,128], cytokines[128,129] and RBP[130].

A panel of 65 urinary biomarkers (DN65) have been identified which distinguished normoalbuminuric patients with diabetes from those with DN. This panel proved sensitive and specific for distinguishing DN from other causes of CKD in both single and multicentre settings[127,131]. CKD273 is a panel of 273 urinary peptides which shows promise as a tool for early detection of DN. First described in 2010, the panel was initially shown to distinguish between CKD of any aetiology and healthy controls with 85.5% sensitivity and 100% specificity[132]. It has also recently been shown to predict adverse outcomes including death or end-stage renal disease in CKD patients[133]. Two further studies have demonstrated the predictive power of CKD273 in identifying diabetic patients at risk of progression to overt DN. In longitudinal samples from a small cohort of 35 diabetic patients Zürbig *et al*[134] showed that application of the classifier to samples from normoalbuminuric subjects up to 5 years prior to detection of macroalbuminuria enabled early identification of those at risk of progression (area under the curve 0.93, compared to 0.67 for urinary albumin). Similarly, Roscioni *et al*[135] applied the classifier to samples from the Prevention of REnal and Vascular ENd-stage Disease (PREVEND) cohort. They compared samples at baseline and 3 years for 44 "progressors" who transitioned from normo-to MA or from micro- to macroalbuminuria to matched controls who did not transition in albuminuria status. Results showed that classifier score at baseline was independently associated with progression of albuminuria[135]. Further to this CKD273 has recently been validated in a multicentre setting. In 165 urine samples obtained from 87 cases of DN and 78 controls at 9 centres worldwide the classifier distinguished cases from controls with high consistency across all centres (areas under the curve ranging from 0.95 to 1.00)[131]. A classification factor cut-off of 0.343 was established in the biomarker discovery cohort to highlight individuals "at risk" of later DN[132] and this has been confirmed by other studies[134,135].

METABOLOMICS

Metabolomics involves the measurement of low molecular weight intermediate and end-products of cellular functions in a biological sample, and has recently emerged as a tool with potential in novel biomarker discovery. The metabolome combines biological information from the genome, transcriptome and proteome, allowing identification of physiological and pathological changes in response to disease processes.As with proteomics, a variety of sample types including serum, plasma, tissue and urine can be analysed in this way[136].

A number of studies have explored the application of metabolomics approaches in kidney disease[136]. For example, in a cross sectional analysis of plasma metabolites using samples from 30 non-diabetic male subjects with CKD stage 2-4, major differences were identified in arginine metabolism, carboxylate anion transport and coagulation pathways with increasing CKD stage[137]. However, this study did not include patients with diabetes and in fact there are a limited number of such studies focussing on diabetic kidney disease. In serum samples from 78 type 2 diabetic participants, a panel of 19 metabolites was identified which could differentiate DN from normoalbuminuria, all of which correlated significantly with albumin creatinine ratio. A model comprising the five best performing markers (including γ-butyrobetaine and symmetric dimethylarginine) resulted in AUC value of 0.927 for diagnosis of DN[138]. Another study using serum samples from patients with DN, normoalbuminuric diabetic patients and healthy volunteers showed significant changes in amino acid and phospholipid metabolism between study categories, as evidenced by alterations in leucine, as well as the sphingolipids dihydrosphingosine and phytosphingosine[139]. Additionally, the application of metabolomics methods to renal cortex samples from streptozocin induced diabetic rats identified an increase in intrarenal organic toxins, including glucuronides, uraemic toxins and others associated with glucotoxicity, which were significantly correlated with 24 h urinary protein levels.Furthermore, treatment with the ACE-inhibitor Fosinopril appeared to block the accumulation of these toxins[140]. There is little published evidence from longitudinal studies to determine the predictive power of these methods for detection of individuals at risk of DN. One such paper published earlier this year described the application of metabolomics methods to urine and plasma samples from the PREVEND study over a median follow up period of 2.9 years. Differences were seen in plasma histidine

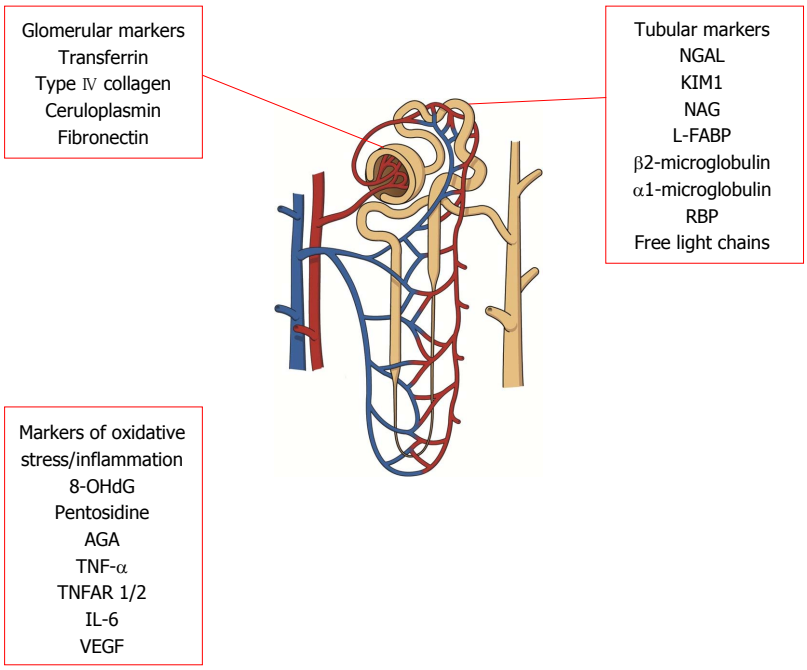

Figure 1 Biomarkers for diabetic nephropathy. NGAL: Neutrophil gelatinase associated lipocalin; KIM1: Kidney injury molecule 1; NAG: N-acetyl-b-d-glucosaminidase; L-FABP: Liver-type fatty acid binding protein; RBP: Retinol binding protein; 8-OHdG: 8-oxo-7,8-dihydro-2'-deoxyguanosine; AGA: α-1-acid glycoprotein; TNFAR 1/2: Tumor necrosis factors-α receptors 1 and 2; IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor.

and butenoylcarnitine, as well as urine hexose, glutamine and tyrosine between individuals who transitioned in albuminuria stage compared to control sample who did not. Adding these metabolites to a predictive model including baseline albuminuria and eGFR appeared to improve risk estimation for transition to macroalbuminuria[141]. However, the complexity of the human metabolome remains perhaps the biggest challenge in translating these techniques into everyday clinical practice (Figure 1).

DISCUSSION AND CONCLUSIONS

DN is a leading cause of end stage renal disease and in combination with the increasing worldwide prevalence of diabetes poses an enormous burden to healthcare systems. UAE is currently the gold standard for detection and monitoring of nephropathy and cardiovascular risk in diabetes; however its predictive powers have limitations and research is focussing on biomarkers which may offer greater sensitivity and earlier detection to facilitate earlier intervention. A degree of caution should, however, be exercised in relation to aggressive early intervention as to date there is little evidence of benefit from these strategies and more intensive RAAS blockade can result in a high incidence of unwanted adverse effects[142,143]. The Randomised Olmesartan and Diabetes MA Prevention study confirmed a significant delay in onset of MA with olmesartan therapy in normoalbuminuric type 2 diabetes patients, but caused controversy regarding increased fatal cardiovascular events in the treatment group[144]. It could be argued that perhaps these studies have not targeted recruitment towards a population at particularly high risk of developing DN and focussing efforts in the direction of these individuals may yield more positive results. Identification of biomarkers to stratify patients according to DN risk may allow randomised controlled trials to focus on the population most likely to derive benefit from early, aggressive intervention.

Markers of glomerular damage show some promise for this purpose. In particular transferrin and type Ⅳ collagen appear to detect glomerular dysfunction at the normoalbuminuric stage although head to head comparative data are lacking. Similarly, given that tubular damage can precede glomerular pathology, markers such as NAG, KIM1 and NGAL are interesting. Evidence also points towards the role of oxidative stress in the pathogenesis of DN, meaning markers such as 8-OHdG and pentosidine merit further investigation. Low grade inflammation and endothelial damage is detectable in the pre-clinical stages of DN, leading to heightened interest in markers such as cytokines and AGA. These too appear to be potentially useful tools in the earlier detection of DN, although again comparative work in relation to UAE would strengthen the case for their use.

The development of new technologies has led to exciting possibilities in the search for ideal biomarkers for DN but, despite the vast number that have been studied, none has so far demonstrated superiority to albuminuria. While biomarker research in the preclinical setting is advancing, none of those biomarkers described above have been validated or are available commercially for clinical use. In addition, none have been described in relation to nonalbuminuric DN, which may reflect a separate disease process. All such potentially interesting markers require further large scale validation in prospective clinical studies to determine whether they can make the transition

from bench to bedside. Projects such as the EU-funded Proteomic prediction and Renin angiotensin aldosterone system Inhibition prevention Of early diabetic nephropathy In type 2 diabetic patients with normoalbuminuria (www.eu-priority.org) study which is currently recruiting, may help to redress this balance.

As the complexities of the biochemical mechanisms underpinning DN continue to be unravelled it is perhaps simplistic to expect that a single biomarker will be sufficient for risk stratification as we move towards predictive and personalised medicine, and as such the shift towards systems biology integrating different technologies into multimarker strategies might provide greater sensitivity and specificity.

PERSPECTIVES

A number of biomarkers show promise as tools for early detection of DN, yet to date none have out-performed microalbumin in larger scale, prospective longitudinal studies. Multimarker approaches such as metabolomic or proteomic methods are particularly appealing as they also offer an insight into the multiple complex pathophysiological processes underlying DN. In order to advance these efforts, cross-omics profiling, large scale biobanking and extended clinical phenotyping will be necessary to derive disease-stage specific models. It should be borne in mind that nonalbuminuric DN is not uncommon and may reflect an alternative underlying disease process, therefore longitudinal studies investigating the performance of biomarkers to identify these individuals early may also be of interest.

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REVIEW

Diabetes mellitus and cellular replacement therapy: Expected clinical potential and perspectives

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Abstract

Diabetes mellitus (DM) is the most prevailing disease with progressive incidence worldwide. Despite contemporary treatment type one DM and type two DM are frequently associated with long-term major microvascular and macrovascular complications. Currently restoration of failing β-cell function, regulation of metabolic processes with stem cell transplantation is discussed as complements to contemporary DM therapy regimens. The present review is considered paradigm of the regenerative care and the possibly effects of cell therapy in DM. Reprogramming stem cells, bone marrow-derived mononuclear cells; lineage-specified progenitor cells are considered for regenerative strategy in DM. Finally, perspective component of stem cell replacement in DM is discussed.

Key words: Diabetes mellitus; Regenerative medicine; Stem cells; Cellular reprogramming; Transplantation

Core tip: Modern approaches to stem cell therapy are discussed a promising component of treatment program in diabetes mellitus. It is important to emphasize that the new technology that is associated with reprogramming of stem cells has a couple of disputes in accordance with the ethical considerations and practical issues. However, the extremely high cost of novel methods toward preventing immune rejection of graft tissue and the high risk of oncogenesis retain their value as major constraints to the implementation into routine clinical practice. The purpose of the review was to summarize and analyze data for existing knowledge and prospects for future researches in the field of regenerative therapy in patients with diabetes mellitus.

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INTRODUCTION

Diabetes mellitus (DM) is the most common endocrine disease, which is considered one of the most important causes of morbidity and mortality worldwide[1]. Type I DM (T1DM) and type 2 DM (T2DM) have different origins, which significantly impact on the ability to achieve adequate glycemic control. T1DM is an autoimmune disease, which is based on absolute deficiency of insulin secretion due to inflammation, necrosis or apoptosis of β cells[2]. In opposite to T1DM, T2DM is defined as predominantly age-related metabolic disease associated with insulin resistance and forming β cell dysfunction that leads to glycemia and different types of metabolic disorders[3]. Although modern treatment of DM1 and DM2 are usually effective and may sufficiently improve clinical status in short-term perspective, it often associates with vascular complications in the long term period that is discussed as a main cause of ischemic lesions of tissues and target-organs damages. All these mediate manifesta-

tion of endothelial dysfunction, retinopathy, nephropathy and cardiomyopathy[4]. The molecular mechanisms that are turned up in resulting of ischemic tissue injury and restoration of tissue perfusion lead to onset and progression of the atherosclerotic damage[5]. As a consequence, atherothrombosis and the exaggerated ischemic tissue injury leading to cardiovascular remodeling mediate increased morbidity and mortality. Overall, DM increases age-related mortality and atherothrombotic related death in two-fold time[6]. It is needed to take into consideration that not all complications of DM appear to be resulting of ischemic causes. As known there are several none-vascular factors associated with an increased risk of manifestation of DM complications, such as not adequate control for hyperglycemia, drug-induced and none-drug-induced hypoglycemia, as well as age-related metabolic comorbidity. It is well known, all they may contribute malignant evolution of DM and negatively relate with poor prognosis and tendency to low effectiveness of therapies. Currently guidelines for diabetic patient treatment focus an opinion of physicians on molecular targets that affects insulin secretion, glucose regulator peptides, hormone regulators, enzymes and transporters. However, it is predisposed that treatment approaches would also mediate improving of hypoglycemia associated with suppression of advanced glycation end products accumulation, decreasing of reactive oxygen species overproduction, improving dyslipidemia and endothelial dysfunction, prevention of atherosclerosis, modification of coexisting cardiovascular risk factors and achieving of adequate control for metabolic comorbidities[7].

Therefore, taking into consideration of particularities of pathogenesis of DM, there are several alternative approaches toward improving of efficacy of contemporary therapy. They are directed to reparation and restoration of β-cell function, improving of metabolic processes by specific way, such as stem cell transplantation[8]. Indeed, therapeutic potency of pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced PCSs in diabetes cure is very promised[9,10]. According novel investigations, several ESCs and induced PSCs lines have to be great differential capacities for DM patients. As expected, they are able to translate into all cell types that have a high ability to differentiate into insulin-secreting β cells with low risk of rejection[10]. However, the data on regenerative DM care obtained several investigators are controversial[11]. Currently we have profound discrepancies in this field between results obtained in animal studies and clinical investigations. On the one hand, unexpected inconsistencies might be related with several strategies of recruitment and maturation of stem cells and using of different types of stem cells. On the other hand, DM patient populations are not uniform that negatively associates with results of stem cells transplantation[12,13]. The purpose of the review was to summarize and analyse data for knowledge and prospects for future researches in the field of regenerative therapy in DM patients.

PARADIGM OF THE REGENERATIVE CARE

The main paradigm of regenerative care bases on new knowledge in DM pathogenesis and several molecular repair mechanisms[14]. Conceived to halt or reverse disease progression, stem cell therapies are applied essentially as adjuvants to standard of care with the goal of furthering an otherwise limited self-renewal capacity of the disease[15].

EFFECTS OF CELL THERAPY

The possibly effects of regenerative therapy might have a many faces and they affect different sides of pathophysiological mechanisms of DM evolution (Figure 1).

The possible approaches for care are: (1) Regeneration of β cell mass and restoring of functional properties of β cell with human stem cells; (2) Stimulation of the endogenous repair mechanisms; and (3) Modulation of metabolic processes in stem cells transplanted through use of appropriate cytokines and growth factors that might be induced direction for further differentiation of stem cells.

However, the innate intimae molecular mechanisms leaded to realize the favorable effects of stem cell transplantation are different (Figure 2).

Regeneration of β cell mass and restoring of functional properties of β cell with human stem cells

The progressive loss of functional pancreatic β cells and insufficient insulin secretion by β cells due to endogenous stimuli are suitable for all forms of DM[8]. As a variant of achieving of increased desired pancreatic β cell mass is allogenic pancreatic islet transplantation. This method is currently considered a most efficient approach for DM treatment in routine clinical practice[16]. However, there are many distinguished strategies to be restoring desired β cell mass from stem cell pools. One of it is strategies is directed to increasing of islet precursor cells from embryonic stem cells under influence of relevant transcription factors (Pdx1, Ngn3, Isl-1, *etc.*), as well as with the use of several extracellular factors. Once a high enough proportion of islet precursors have been obtained there is a need for cell-lineage selection in order to purify the desired cell pools[17]. More detail cellular mechanisms for stem cell reprogramming aimed regeneration of pancreatic β-cell mass are described in excellent review represented by Pandian *et al*[18]. It has emphasis that there is transplantation of exogenous pancreas/islets or artificial islets, enhanced proliferation and maturation of endogenous β cells, prevention of β-cell loss, or fortified renewal of β-like-cell populations from stem cell pools and non-β-cell sources[19,20]. Results of recently performed investigations have been revealed that there are serious limitations regarding efficacy and safety of various types of cell replacement therapies aimed restoration of functional β-cell sources[21]. However, when several strategies were compared each other the restoring

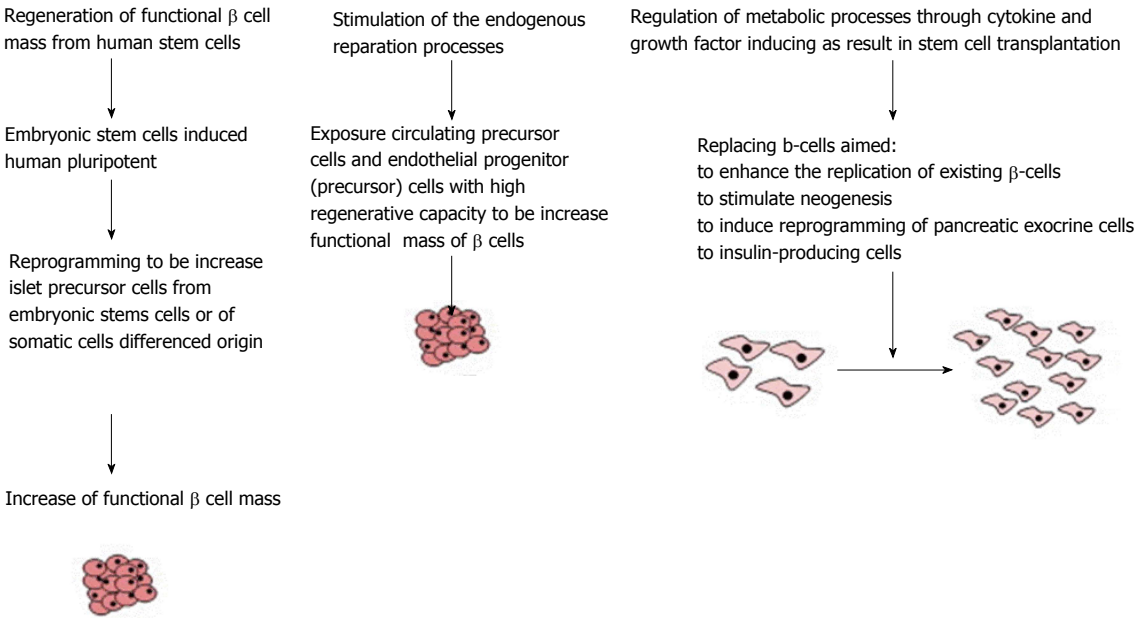

Figure 1 The possible approaches of cell therapy in diabetes patients.

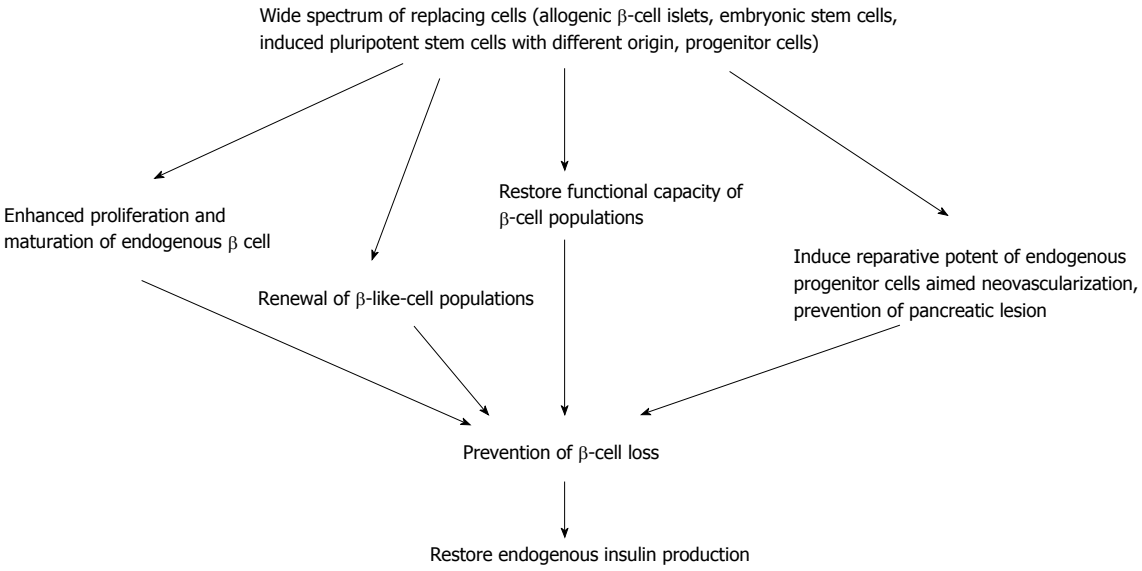

Figure 2 The potent molecular mechanisms that lead to realize an effect of cell therapy in diabetes.

of functional β cell mass from human stem cells for cure in T1DM appears to be most promising approach[22]. One of explanation of this phenomenon was use of specific methods and techniques for generating of stem cells from different source[23].

As known there are at least two practically important sources for human pluripotent stem cells: (1) Deriving of ESCs from blastocysts that were created *in vitro*; and (2) Induced PSCs generated from different cell lineages of somatic cells using reprogram methods[17-19].

As we can see, ESC deriving is an attractive area of scrutinizes. Now there are at least two clinical trials that were recently finished and the results obtained have let to approve the performing technique for further clinical practice. However, the closely discussion with various specialists are required to be understand whether will the results have serious clinical value or not[24]. Overall, it is not exactly known whether will different cell lineages of embryonic or adult stem cells have high potency to differentiation into β-like cells or not. Moreover, we cannot say that only isolated restoring of the original insulin secretory activity of the transferred cells is expected. It is needed to take also into consideration that immunomodulatory effect of cells transferred affected other tissue cells may be possible and that this phenomenon may lead to autoimmune destruction of previously transplanted cells and other tissue cells[21].

Because human induced PSCs appear to be highly similar to human ESCs, novel technology based on reprogramming of various originated PSCs is discussed

as one of the most promising technique[25]. Now it is known that PSCs may be successfully derived from various human somatic cells, such as dermal fibroblasts and keratinocytes[26]. Therefore, autologous pancreatic islets may be differentiated from induced PSCs that derived from DM subjects using integrating retroviral vectors that integrate into the host genome and after then it may replace to donor[27-29]. Importantly, that use embryonic cells in this case is not required. Based on the results of the contemporary investigations, it is possibility emphases that induced PSCs that have been derived from DM subjects with helping of various trans-differentiation techniques are not similar on their biological safety[27-30]. There are needing for continuously investigations of more representative technologies that may let us sufficiently improve of biological hazardless around strategy based on induced PSC transfer. However, before clinical implementation of induced PSCs transplantation there is required to perform fundamental investigations related the specificity, efficiency, kinetics, and biological safety of novel methods of cell reprogramming. Despite results of controlled studies in this field are limited, novel approaches regarding improve and change the induced PSC process promise to be more successful than previous[31]. Currently there are some transcription factors (molecular factors, vectors, various small molecules) that might be useful for improving functionality of induced PSCs before replacement. All these may increase an attractive of trans-differentiation technique to derive one somatic cell type to another patient-specific cell through step associated with induced PSCs obtained[29]. Results of the recently studies have been found that using transcription factors for trans-differentiation of induced PSC into patient-specific cells may open a new era of regenerative medicine. The use of different types of somatic cells with trans-differentiation technology is consider an important approach for improving plastic of induced PSC reprogramming and as serious extend of possibilities for increasing efficacy and biological safety of regenerative medicine[29,31]. Finally, irrespective several limitation of clinically-based evidences of implementation of trans-differentiation on routine clinical practice, it is required to accumulate efforts toward summarize of knowledge about novel method of induced PSC transcription.

The contemporary investigations regarding clinical using of insulin-producing surrogate cells derived from ESCs have been revealed controversial results. This would be related with uniformness in transcription factors use and in the sufficiently differentiation affected techniques of ESC deriving. However, there is no consensus on common standard protocols regarding clinical approaches mentioned above[32,33]. Despite the contemporary statements are required improvement, they present requirement about uniform technology regarding differentiation methods of deriving pancreatic progenitor cells from pluripotent cells[25]. Therefore, another source of deriving of autologic insulin-producing β-cells is tested. Indeed, human bone marrow mesenchymal stem cells (hBM-MSCs) might be considered a source for restoring functionally capacity of β-cell and also probably islet-like clusters that leads to β-cell mass increasing[20]. It is expected that microenvironmental of hBM-MSCs may improve trans- differentiation this type of cell into insulin-produced β-cells. There are data that platelet-rich plasma might be useful for increasing of differentiation capacity of the hBM-MSCs[34]. Moreover, it has been postulated that hBM-MSCs probably would be considered more optimal candidates for further clinical implementation when compared with induced PSC, while this predisposition is required strong and continuous investigations.

Stimulation of the endogenous reparation processes

There are evidences that circulating precursor cells and endothelial progenitor (also known as precursor) cells (EPC) are reduced in DM with advanced complications such as critical limb ischemia, peripheral neuropathy and neuropathic diabetic foot. It is expected that EPC labeled $CD34^+KDR^+$ and $CD31^+CD133^+$ could have not only a sufficient prognostic value, but and therapeutic significance in DM patients with neuropathic and ischemic lesions[35]. The expected effect of EPC associates with stimulation of the endogenous repair process in the field of the endothelium that may lead to improving of clinical evolution of DM. It is needed to emphases the signaling pathways that lets EPC to differentiate into functional β-cells and mature endothelial cells are still poorly understood and their clinically potency is being be currently unresolved[36].

The strategy of regulation of metabolic processes with stem cells

Some alternative approaches for replacing β-cells include follow principal ways toward to enhance the replication of β-cells, stimulation of neogenesis of the tissues affected DM-related injury, and reprogramming of autologic pancreatic exocrine cells to patient-specific insulin-producing cells. The contemporary approaches based on various type stem-cell deriving might also be useful for effective modulation of the immune system response in T1DM patients. It is also possible the problems of obesity and insulin resistance appearance in T2DM could resolve with immune system response modulation through patient-specific insulin-producing cells transfer[19]. It is predisposed that such approaches may lead to increased efficacy regeneration of pancreatic β-cell mass and functional activity of restoring β-cells[17,18]. Another potential factor could be mediated the effects of stem cells are cytokine and growth factor, but their clinically importance in DM patients is not still understood.

RESULTS OF PRE-CLINICAL STUDIES OF STEM CELL-BASED THERAPY

Early experience in the treatment of diabetes employs stem cells in their native state, as well as unfractionated or enriched in progenitor subpopulation cells, but next generation of cell delivery such as reprogramming stem

Table 1 Summary preclinical data among stem cell transplantation in diabetic animals

Type of cell replaced	Positive effect expected	Negative effect expected
Embryonic stem cells	Direct effect: Differentiation into functional insulin-producing cells Indirect effect: Improving of the fasting blood glucose due to restore the function of islet β cells Decreasing of blood lipid levels Increasing of serum C-peptide level Prevention of free-radical induced oxidative stress injury of beta-cells Improving of pancreatic microcirculation	Ethical problems Rejection High frequency of autoimmune-mediated destruction of the β cells and other autoimmune reactions High immunogenency Malignancy Potential tumor mediated effect
Pluripotent stem cells	Direct and indirect effects: See mentioned above	High frequency of rejection High immunogenency Low frequency of autoimmune-mediated destruction of the β cells and other autoimmune reactions Potential tumor mediated effect
Bone marrow derived mesenchymal stem cells	Direct and indirect effects: See mentioned above	Low frequency of autoimmune-mediated destruction of the β cells Moderate immunogenency Potential tumor mediated effect Low frequency of rejection
Adipose-derived stem cells	Direct and indirect effects: See mentioned above	Extremely low incidences in comparison with bone marrow derived mesenchymal stem cells of rejection, potential tumor mediated effect and autoimmune-mediated destruction of the β cells

cells, bone marrow-derived mononuclear cells; lineage-specified progenitor cells are considered more perspective (Table 1).

Reprogramming stem cells

A new era in reprogramming of stem cells is related with techniques of therapeutic cloning. Recently it has been reported to have a high potency in DM treatment[37]. Now there are essential requirements of a material designed as stem cells differenced origin recruited for further reprogramming process[38]. These include ESCs and multipotent adult stem/progenitor cells derived from a wide range of tissues (pancreas, intestine, liver, bone marrow, brain, *etc.*)[39]. There are various evidence for using of recombinant proteins or pharmacologic drugs to induce and mediate the reprogramming process[40,41]. The strategic approaches include follow important direction affected development of generating methods and technologies that associates with non-integrating, non-viral, and non-genetic techniques toward induced PSCs deriving[41]. There are some basic conditions for pluripotency determination that have been identified *in vitro*, and aimed at specific types of somatic cells[42]. The high quality review presented by Hindley *et al*[43] that is devoted current understanding of possible interrelationship between the core cell cycle machinery and the maintenance of pluripotency in ESCs and induced PSCs. However, there are advantages of therapeutic cloning affected the potential of cells originated from non-β-cell and related with avoiding of the autoimmune response after transplantation[44]. Despite there is a high similarity of different types of ESCs, effectiveness of reprogramming methods is low and successful result of stem cell culturing appears in 0.01%-0.1% cases[26]. These facts are considered a cause for design of stem cell bank in short-term perspective[45].

Although tremendous clinical effects of stem cell transfer are related with induced PSC transplantation, majority experts have been believed that differentiation of self-renew autologic somatic cells into specific patient-related cells are more desirable approach then ESCs and induced PSC transplantation[46]. However, fully pluripotency is remained available capacity for various lines of human induced PSC[37]. Little known whether these advances for new treatment care in DM patients will preserve[47,48]. Currently new lines of PSC might be powerful for mediation of the molecular mechanism regulation affected the reprogramming process of stem cells different origin[49].

Bone marrow derived mesenchymal stem cells transplantation

Although there is significant progress in the development of safety in turn of clinical implementation of the first derivation of ESCs and induced PSCs, transgene-free induced PSC methods of reprogramming technology have to be attractive as the best technique for culturing of pluripotent stem cells[50]. Cell therapy based on mesenchymal stem cell (MSC) transplantation is considered an effective in the treatment of DM with higher level of safety and tolerability when compared with ESCs. Bone marrow mesenchymal stem cells (BMSCs) have individual particularities that appear to be self-renewing capacity. Therefore, BMSCs represent multipotent activity and may migrate to appropriate pathological sites for realizing their therapeutic potency. The successful BMSC transplantation was presented in animal model of T2DM and it was associated with significantly improving of the fasting glucose and decreased atherogenic circulating lipids in blood. Other biological markers of cardiovascular and metabolic risk were modulated also after transfer of BMSCs. Indeed, circulating C-peptide levels were significant-

ly increased in resulting of BMSCs transplantation[51]. El-Tantawy *et al*[52] reported that autologous BMSCs appear a significantly potency to prevention of tissue alterations in animals with DM. This effect was probably associated with attenuation of the alloxan-induced oxidative stress. Authors have believed that BMSCs demonstrate rigorous ability for differentiation into functional insulin-producing β-cells and that therapeutic effect of BMSCs may allow achieving an adequate control for hyperglycemia, improve hyperlipidemia, and suppress oxidative stress. All these mentioned above may be helpful in the global strategy toward prevention of DM-related complications.

Tang *et al*[53] investigated the effect of transplantation of autologous BMSCs in streptozotocin-induced DM pigs. The results obtained in the animal model have been showed that transplantation of autologous BMSCs may help to reverse a streptozotocin-induced DM. Moreover, after transplantation the autologous BMSCs leaded to restoring of blood glucose levels, improving of glucose tolerance test and pancreatic microcirculation, increasing of circulating insulin and C-peptide, as well as the number of islets was significantly increased. Obviously these data suggested that autologous BMSCs implantation might be useful as alternative strategy of DM. Overall, majority investigators have been concluded that the transplantation of BMSCs aimed alternative treatment of DM added to conventional strategy is safe and effective[52,53].

Limitation of the cell therapy in DM

There is wide spectrum of serious limitations for transplantation of the stem cell. The main obstacles affected success of the strategy in T1DM is autoimmune-mediated destruction of the transplanted β-cells and pancreatic islets[54]. One of the possible causes leaded to low efficacy of stem cell transplantation is cellular damage during the isolation process and donor shortages[55]. All these stimulate efforts for creating of novel techniques for increase transplantation efficacy by co-culturing single primary islet cells with adipose-derived stem cells (ADSCs). Now it has suggested that ADSCs may have a sufficient potency to islet cell protection from damage during culturing. Despite this expectation, no significant evidences that the ADSC use improve survival of islet cells and their functionality prior to transplantation procedure. In this context many investigators point that culturing technique is crucial for efficacy of xenotransplantation procedure. Indeed, *in vivo* experiments with involving xenotransplantation of microfiber-encapsulated spheroids into a mouse model of DM have found that co-culture-transplanted mice lead to higher glucose metabolism modulation when compared with mono-culture-transplanted mice. The novel method for culturing islet spheroids were tested by Jun *et al*[55]. Investigators concluded that new technique is potentially over helmed the traditional technologies in turn of cell shortages. Moreover, islet spheroids culturing may probably consider a biological artificial pancreas. Currently, both cell source, ESC and induced PSC, allow achieving a high levels of insulin-produced β-cell differentiation, but due to ethical issues and the potential malignancy risk after transplantation clinical use of these approaches are limited Next alternative strategy to be overcome the such seriously obstacles mentioned above is attempts to use pancreatic epithelial cells that may also represent capacities for differentiation into patient-specific insulin-produced β-cells. However, there are major reasons for limitation in clinical implementation of pancreatic epithelial cells due to their high immunogenency. Finally, induced PSCs, ADSCs, and BMSCs are currently discussed the great promise for regenerative medicine in DM field.

EXPECTANCIES OF STEM CELL-BASED THERAPY IN DIABETIC PATIENT POPULATIONS

The expectations that cell therapy may appear new strategy approach for restoring of β-cell mass and their functionality is based on the results of recent investigations. They have been indicated that full glycemic control may be achieve after replacement of autological β-cells and induced PSCs[56]. The pre-clinical studies in support of regenerative paradigms in DM have been tested in different clinical settings with using of various stem cell culturing[57]. It is traditional techniques for human ESCs culturing are incompatible with the generation of genetically diverse, patient- or disease-specific stem cells[58]. The basic data among stem cell-based therapy in diabetic patient population are presented in Table 2. However, the overall efficiency of the conversional nuclear transfer is very low and the safety issue remains a major concern for induced PSCs implementation in various DM patient populations[59]. Overall, the results of the recent studies are controversial due to lack uniformity of design and protocols related techniques of the cell isolation and delivery methods[33]. Moreover, accordingly opinion Soejitno *et al*[26], the implementation of the stem cell in the routine clinical setting is limited due to risk of malignancy, autoimmune response and rejection of the transplanted cells. Indeed, the allogeneic immune rejection of human ESC-derived cells is considered the main cause of efficacy limitation in recipients[23]. This important problem might be attenuate by implementation of the novel technology affected nuclear reprogramming of induced PSCs in DM patients. However, despite many significant advances novel technological approaches recent clinical studies did not shown superiority new treatment when compared with traditionally methods based on induced PSCs therapy[23]. Finally it is required novel clinical investigations with greater statistical power to be resolving of the situation around efficacy of various methods of the cell therapy in DM[60].

FUTURE PERSPECTIVES OF REGENERATIVE THERAPY

The ability to interconvert terminally differentiated cells

Table 2 The basic data among current and completed stem cell-based investigations in diabetic patient population

Title of the study/ClinicalTrials.gov identifier	Phase	*n*	Gender	Age group	Cell type	Interventions	Results
Tissue distribution of F18-FDG labelled autologous bone marrow derived stem cells in patients with type 2 DM (NCT01694173)	Phase 2/3	28	Both gender	Adult/ senior	Stem cell harvest	Splenic artery transplantation *vs* placebo	No data, current study
Efficacy of autologous bone marrow derived stem cell transplantation in patients with type 2 diabetes mellitus (NCT00644241)	Phase 2	10	Both gender	Adult/ senior	Stem cell harvest	Angiographic transplantation of stem cells	No data, current study
A pilot study on transplantation therapy using autologous bone marrow mononuclear cells and umbilical cord mesenchymal stem cells in patients with type 1 diabetes mellitus (NCT01143168)	Phase 1	24	Both gender	Adult	Autologous bone marrow mononuclear cells and umbilical cord mesenchymal stem cells	Angiographic transplantation of stem cells	No data, current study
A open labeled and self controlled, safety/efficacy assessed pilot study on transplantation therapy using bone marrow mesenchymal stem cells for insulin resistance of type 2 diabetes mellitus (NCT01142050)	Phase 1	24	Both gender	Adult	Mesenchymal stem cells	Angiographic transplantation of stem cells	No data, current study
Autologous hematopoietic stem cell transplantation in type 1 diabetes mellitus (NCT01121029)	Phase 1/2	15	Both gender	2-35 yr	Autologous hematopoietic stem cell	Transplantation	Beta cell function was increased in all but 1 patient and induced prolonged insulin independence in the majority of the patients
Autologous bone marrow mononuclear cell infusion with hyperbaric oxygen therapy in type 2 diabetes mellitus (NCT00767260)	Phase 1/2	82	Both gender	45-65 yr	Autologous bone marrow mononuclear cell	Autologous bone marrow mononuclear cell Infusion *vs* standard medical therapy	No data, current study
Phase 1 and 2 study of the use of human adipose derived mesenchymal stem cells as regenerative therapy in diabetic patients with critical limb ischemia (NCT01257776)	Phase 1/2	36	Both gender	18-85 yr	Autologous adipose derived mesenchymal stem cells	Intra-arterial administration through a selective cannulation of target common femoral artery *vs* no intervention	No data, current study
Efficacy of autologous bone marrow derived stem cell transplantation in patients with type 2 diabetes mellitus (NCT01065298)	Phase 1/2	30	Both gender	30-75 yr	Autologous Bone marrow derived stem cell	Injection into superior pancreatico duodenal artery *vs* standard combined medical therapy	No data, current study
Study on induced wound healing through application of expanded autologous bone marrow stem cells in diabetic patients with ischemia-induced chronic tissue ulcers affecting the lower limbs (NCT01065337)	Phase 2	30	Both gender	18-80 yr	Bone marrow stem cells	Intraarterial administration *vs* standard of care wound treatment according guideline of the American Diabetes Association	No data, completed study
Phase 2 study of autologous stem cell and hyperbaric oxygen therapy in type 2 diabetes mellitus (NCT01786707)	Phase 1/2	2	Both gender	45-65 yr	Autologous stem cells	Autologous stem cells and hyperbaric oxygen therapy *vs* No Intervention	No data, completed study
Reversal of type 1 diabetes in children by stem cell educator therapy (NCT01996228)	Phase 1/2	20	Both gender	6-14 yr	Human Cord Blood-derived multipotent stem cells	Apharesis and stem cell educator therapy	No data, current study
Phase 2 study of stem cell educator therapy in type 1 diabetes (NCT01350219)	Phase 2	100	Both gender	14-60 yr	Human cord blood-derived multipotent stem cells	Apharesis and stem cell educator therapy	No data, current study
A trial of high dose immunosuppression and autologous hematopoietic stem cell support *vs* intensive insulin therapy in adults with early onset type 1 diabetes mellitus (NCT01285934)	Phase 1/2	30	Both gender	16-35 yr	Autologous hematopoietic stem cell	Autologous hematopoietic stem cell transplantation *vs* intensive insulin therapy	No data, current study
Stem cell educator therapy in type 2 diabetes (NCT01415726)	Phase 1/2	25	Both gender	14-65 yr	Human cord blood-derived multipotent stem cells	Stem cell educator used for the isolation and purification of cord blood stem cells. No comparator	No data, current study
Safety and efficacy study of umbilical cord/placenta-derived mesenchymal stem cells to treat type 2 diabetes (NCT01413035)	Phase 2	30	Both gender	18-80 yr	Human umbilical cord/ placenta-derived mesenchymal stem cells	Human umbilical cord/placenta-derived mesenchymal stem cells iv infusion + oral hypoglycemic drugs, insulins or their combination vs oral hypoglycemic drugs, insulins or their combination	No data, current study
Open study to evaluate the safety and efficacy of autologous mesenchymal stem cells in treatment of recently diagnosed patients with type 1 diabetes mellitus (NCT01068951)	Phase 2	20	Both gender	18-40 yr	Autologous mesenchymal stem cells	Autologous transplantation of the patients own mesenchymal stem cells (approximately 2×10^6 cells/ kg body weight) intravenously.	No data, completed study

Umbilical cord mesenchymal stem cells and liraglutide in diabetes mellitus (NCT01954147)	Phase 1/2	100	Both gender	35-65 yr	Umbilical cord mesenchymal stem cell	Umbilical cord mesenchymal stem cell infusion combined with liraglutide *vs* liraglutide	No data, current study
Umbilical mesenchymal stem cells and mononuclear cells infusion in type 1 diabetes mellitus: a randomized controlled open-label study (NCT01374854)	Phase 1/2	44	Both gender	18-40 yr	UC-MSCs	1×10^6/kg UC-MSCs is infused through pancreatic artery along with mononuclear cells by interventional therapy and another same dose of UC-MSCs is administered one week post-intervention	No data, current study
Autologous transplantation of mesenchymal stem cells for treatment of patients with onset of type 1 diabetes (NCT01157403)	Phase 2	80	Both gender	10-40 yr	Autologous bone marrow mesenchymal stem cells	Autologous transplantation of bone marrow mesenchymal stem cells (approximately 2.5×10^6 cells/kg body weight) intravenously	No data, current study
A phase II, multicenter, randomized, double-blind, placebo-controlled study to evaluate the safety and efficacy of prochymal® (*ex vivo* cultured adult human mesenchymal stem cells) for the treatment of recently diagnosed T1DM (NCT00690066)	Phase 2	60	Both gender	12-35 yr	*Ex vivo* cultured adult human mesenchymal stem cells	Intravenous infusion of *ex vivo* cultured adult human mesenchymal stem cells	No data, current study
A randomized, controlled, parallel design, safety and efficacy study of granulocyte colony stimulating factor mobilized autologous peripheral blood mononuclear cell therapy in subjects with diabetic limb ischemia (NCT00922389)	Phase 1/2	36	Both gender	18-65 yr	Peripheral blood derived mononuclear cells	Implanting stem cells derived from peripheral blood after G-CSF mobilization	No data, current study
Phase 1/2 study: treatment of patients with diabetic foot complications with allogeneic bone marrow derived mesenchymal stromal cells (NCT01686139)	Phase 1/2	10	Both gender	18-81 yr	Cultured Bone Marrow Mesenchymal Stromal Cells (BM-MSCs) from allogeneic donors or autologous BM-MSCs	Multiple injections of ABMD-MSC cells ($10\text{-}20 \times 10^6$ cells)	No data, current study
Autologous hematopoietic stem cell transplantation for the treatment of limb ischemia and diabetic neuropathy in patients with diabetes mellitus type 2: a randomized controlled trial (NCT00730561)	Phase 1/2	20	Both gender	18-74 yr	Hematopoietic stem cell (totipotential, hematopoietic or endothelial lineages)	Intramuscular application of CD34+ hematopoietic stem cells (with a minimum of 2 million CD34+ cells/kg) into the gastrocnemius muscles after stimulation with subcutaneous filgrastim 600 micrograms/kilogram a day for 4 d	No data, completed study

could serve as a powerful tool for cell-based treatment of DM. Using wide spectrum of reprogramming factors investigators could activate *de novo* conversion of intestinal epithelial cells into insulin-produced β-like cells[61]. Authors concluded that the intestine is an accessible and abundant source of functional insulin-producing cells. This fact is intriguing and may have a serious clinically significant value.

The other way is the transplantation several types of stem cells derived from adult cells of pancreas, bone morrow, liver, and cells various originated is under consideration[57]. The lack of transplantable pancreatic islets is a serious problem that affects the treatment of patients with T1DM. The new strategy of regenerative medicine suggests that these obstacles are potentially to be overcame and that the aim of this approach is transformation of any somatic cells into insulin-produced patient-specific β-cells[57]. Contemporary biological and analytical techniques help us to predict the transcription factors that are needed for β-cell regeneration and restoring of the β-cell mass[62]. The transcription factors mediate β-cell renewing with diverse culturing methods[63]. In this context novel cellular strategies toward reprogramming may have better clinical prospects[64,65]. In has been expected that small molecules might be successful to be inducing pancreatic β-cell modification. Recently, a synthetic DNA-based small molecule triggered targeted transcriptional activation of pancreas-related genes to suggest the possibility of achieving desired cellular phenotype in a precise model[66]. Besides providing new β-cells, cell therapy also has to address the question on how to protect the transplanted cells from destruction by the immune system *via* either allo- or autoimmunity[66,67].

In conclusion, stem cell replacement as a perspective component of therapy for DM has received much attention. Importantly, novel technologies for reprogramming of stem cells, such as somatic cell nuclear transfer, meet several ethical and practical concerns. Other significant obstacles remain high cost, methods to prevent immune rejection of grafted tissues, and suppression of the risks of tumorigenesis. For overcoming these obstacles probably more scientific discussions around ethical principles, methods of culturing of stem cells, routine clinical procedures and protocol evaluation, as well as more clinical investigations in this field are required.

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REVIEW

Linking uric acid metabolism to diabetic complications

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Abstract

Hyperuricemia have been thought to be caused by the ingestion of large amounts of purines, and prevention or treatment of hyperuricemia has intended to prevent gout. Xanthine dehydrogenase/xanthine oxidase (XDH/XO) is rate-limiting enzyme of uric acid generation, and allopurinol was developed as a uric acid (UA) generation inhibitor in the 1950s and has been routinely used for gout prevention since then. Serum UA levels are an important risk factor of disease progression for various diseases, including those related to lifestyle. Recently, other UA generation inhibitors such as febuxostat and topiroxostat were launched. The emergence of these novel medications has promoted new research in the field. Lifestyle-related diseases, such as metabolic syndrome or type 2 diabetes mellitus, often have a common pathological foundation. As such, hyperuricemia is often present among these patients. Many in vitro and animal studies have implicated inflammation and oxidative stress in UA metabolism and vascular injury because XDH/XO act as one of the major source of reactive oxygen species Many studies on UA levels and associated diseases implicate involvement of UA generation in disease onset and/or progression. Interventional studies for UA generation, not UA excretion revealed XDH/XO can be the therapeutic target for vascular injury and renal dysfunction. In this review, the relationship between UA metabolism and diabetic complications is highlighted.

Key words: Uric acid; Xanthine dehydrogenase/xanthine oxidase; Diabetes mellitus; Diabetic complications; Xanthine oxidase inhibitor; Metabolism

Core tip: Uric acid (UA) is derived from essential metabolism, and UA metabolism is becoming a novel risk and interventional factor of lifestyle-related diseases in this obesity-prone era. The relationship between UA metabolism and diabetic complications is highlighted in this review and supposed molecular mechanisms are mentioned.

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URIC ACID METABOLISM

Gout, which is caused by increased serum uric acid (SUA) levels, is becoming one of the most prevalent lifestyle-related diseases. According to the National Livelihood Survey in Japan, 874000 people go to hospital for gout in 2004. This constitutes an increase of 3.4 times compared with 1986. Higher prevalence of metabolic syndrome (MetS) is one possible cause for this increase in gout cases, as both the reduced excretion and increased production of UA have been suggested to be associated with MetS. Increased visceral adiposity also causes MetS. In mice, evidence exists that UA is secreted from bloated adipocytes[1]. No studies in humans have confirmed this finding yet.

Uric acid (UA) (2,6,8-trihydroxypurine, C5H4N4O3) is a purine derivative. UA metabolism is a type of nucleic acid metabolism metabolizing purine and its derivatives (adenine, and guanine). Phosphorus oxidation of adenine and guanine (resulting in ATP and GTP) and UA production are essential for many physiological functions. For example, high fructose consumption cause hyperuricemia.

FACTORS THAT DEFINE SERUM URIC ACID LEVELS

SUA levels are determined by a balance between UA production and excretion. At present, no method for detecting the UA production rate is available in humans. Instead, UA production are indirectly speculated through SUA level and urine excretion. The rate-limiting step of UA production is an enzymatic reaction of the xanthine dehydrogenase/xanthine oxidase (XDH/XO) enzyme that oxidizes hypoxanthine-xanthine into UA. Human XDH/XO was cloned in 1993 by Richard[2]. It is expressed in the liver and small intestine of XDH/XO-rich parenchyma cells[3] and is thought to be the major source for SUA. The enzyme is also expressed in adipose tissue, the vascular endothelium, and macrophages, all of which are implicated in lifestyle-related diseases[4]. The UA production rate is based on the amount of substrate and/or XO activity. Since the generation of reactive oxygen species (ROS) depends on XO activity, XO is one of the major sources of oxidative stress in cells along with nicotinamide adenine dinucleotide phosphate oxidase, myeloperoxidase, lipoxygenase, and nitric oxide synthase[5].

The kidney is an important regulator of circulating UA levels and is responsible for 60%-70% of total body UA excretion[6]. The remaining UA is secreted into the intestine, followed by bacterial uricolysis[6]. UA excretion in the kidney consists of urate secretion and reabsorption, and earlier research suggests the involvement of hyperfiltration[7]. UA apical transporters [uric acid transporter 1, organic anion transporter 4 (OAT4), OAT10, sodium-coupled monocarboxylate transporters 1/2, and Na^+-dicarboxylate cotransporter (NaDC1)], which are expressed in the nephron lumen are implicated in the reabsorption process. The role of basolateral transporters in proximal tubular cell is not clarified except for glucose transporter type 9 (GLUT9). During the secretion process, UA is transported into proximal tubular cells *via* OAT1/3 and/or NaDC3 and then secreted by human uric acid transporter, Na^+-phosphate cotransporter (NPT), ATP-binding cassette sub-family G member 2 (ABCG2), and/or ATP-binding cassette sub-family C member 4. Ninety percent of UA filtered by the kidney is reabsorbed[6]. In the intestine, ABCG2 is responsible for about 50% of UA efflux[8-10].

There are many studies about genetic variations exhibiting hyperuricemia. Among genes introduced above, variants of GLUT9 (SLC2A9)[11,12], NPT (SLC17A1)[13], ABCG2 (BCRP) variant[14], are well established and proved to be important in hyperuricemia as a result of decreased extra-renal urate excretion. Genome-wide association study is applied for detecting loci affecting serum UA level. Recent report identified 18 new loci (18 new regions in or near TRIM46, INHBB, SFMBT1, TMEM171, VEGFA, BAZ1B, PRKAG2, STC1, HNF4G, A1CF, ATXN2, UBE2Q2, IGF1R, NFAT5, MAF, HLF, ACVR1B-ACVRL1 and B3GNT4) associated UA concentrations[15]. Not only transporters, but also transcriptional factors, signaling receptors, enzymes are involved in serum UA level.

UA LEVELS IN TYPE 2 DIABETES MELLITUS AND METS

Table 1 shows association between life-style related diseases and UA metabolism[16-24]. Distinguishing cause and effect is difficult; some diseases raise SUA level, but UA affect disease onset or progression.

In patients with diabetes, the SUA level is low due to increased urate clearance[20,25]. In these patients, hypouricemia is associated with glycosuria[26], decreased metabolic control, hyperfiltration, and a late onset of disease, while elevated SUA is a feature of hyperinsulinemia or insulin resistance[7]. Type 2 diabetes mellitus (T2DM) is a risk factor for nephrolithiasis and has been associated with UA stones[27]. It has been suggested that patients with UA stones, especially if overweight, should be screened for T2DM or MetS[28]. The rate of obesity is increasing in Asia as well as in Western countries[29], and hyperuricemia will increase in patients with T2DM. Novel class of anti-diabetic agent, sodium glucose cotransporter 2 inhibitor lowers serum uric acid through alteration of uric acid transport activity in renal tubule by increased glycosuria[21,30].

T2DM ONSET AND UA LEVELS

Besides age, race, family history of diabetes, body mass index (BMI), glucose intolerance, and MetS, SUA levels have been suggested to be associated with T2DM risk[31]. If elevated SUA levels play a causal role in T2DM, SUA might also indirectly affect the prevalence of diabetic complications. The diabetogenic action of UA was reported in 1950[32]; however, its physiological mechanism is not yet known. SUA levels affect insulin resistance[19] and show a significant correlation with risk factors for MetS (high BMI, blood pressure, fasting plasma glucose, and triglyceride levels) and low HDL cholesterol values[19,31,33,34]. Moreover, high SUA levels were shown to predict MetS in a Japanese cohort[35]. We previously reported an association between inflammation, macrophage activation, and SUA production *via* XDH/XO activation in an animal model[36]. In summary, a link between SUA and insulin resistance has repeatedly been shown, and UA itself reportedly plays an important role in the exacerbation of insulin resistance[37].

Table 1 Association between life-style related diseases and uric acid metabolism

Diseases/status	SUA level	UA production	Focus 1	UA excresion	Focus 2
T2DM	High/low				
Glucosuria	Low			Up	Glomerulus
Insulin resistance	High			Down	Proximal tubule cell
Use of SGLT2 inhibitor	Low			Up	
Retinopathy		Up	Vitreus		
MetS	High	Up	Adipocyte/liver?	Down	Proximal tubule cell
CKD	High	Up	Vascular endothelial cell/inflammatory cell	Down/up	Kidney/intestine
Hypertension	High	Up			
Atherosclerosis		Up	Vascular endothelial cell/inflammatory cell		
Reperfusion injury		Up	Vascular endothelial cell		
Heart failure		Up	Inflammatory cell		
Fructose intake	High	Up	Liver	Down	
Sodium intake	High			Down	
Thiazide administration	High			Down	Proximal tubule cell

UA: Uric acid; SUA: Serum uric acid; T2DML: Type 2 diabetes mellitus; CKD: Chronic kidney disease; MetS: Metabolic syndrome.

DIABETIC COMPLICATIONS AND UA LEVELS

SUA independently predicted the development of vascular complications, both retinopathy and nephropahy and coronary artery calcification in type 1 diabetes study by Bjornstad *et al*[38]. The following section discusses the relationship between SUA levels and each diabetic complication.

Neuropathy

Diabetic neuropathy is occasionally the initial manifestation of disease in T2DM patients[39]. It leads to chronic pain, numbness, and substantial loss of quality of life. The prevalence of diabetic peripheral neuropathy shows a significant correlation with increased UA levels[40]. Several studies demonstrated that, when controlled for confounding factors such as age, gender, BMI, renal function, and/or diabetic duration, SUA levels were high in patients with diabetic polyneuropathy and sudomotor dysfunction[41-43].

The pathophysiology of diabetic neuropathy is not completely understood, and multiple metabolic imbalances underlie the development of diabetic neuropathy[44]. Hyperglycemia, dyslipidemia, and cardiovascular dysfunction are all independent risk factors for neuropathy. Probable etiologic factors include the polyol pathway, non-enzymatic glycation, free radicals, oxidative stress, and inflammation. Oxidative stress and inflammation are involved in XDH/XO activity. It is therefore speculated that UA generation by XDH/XO plays a role in diabetic neuropathy.

Diabetic retinopathy

The presence of diabetic retinopathy (DR) is associated with visceral fat accumulation and insulin resistance in T2DM patients[45]. An earlier report found no significant difference in UA levels between patients with or without retinopathy[46], but several recent studies showed a significant increase of UA-related metabolites levels in DR compared to T2DM[47]. SUA concentration was shown to be associated with an increased severity of DR over a three-year period in patients with T2DM. Cox regression analysis showed that patients with SUA levels in the third (5.9-6.9 mg/dL) and fourth ($\geq$ 7.0 mg/dL) quartiles had increased hazard ratios for DR when compared with patients with SUA in the first quartile ($<$ 4.9 mg/dL)[48]. Furthermore, vitreous UA and glucose concentrations were higher in proliferative than in non-proliferative DR. Focal UA production in the vitreous is thought to be involved in the pathogenesis and progression of DR[49].

Nephropathy

Shichiri *et al*[50] showed that glomerular hyperfiltration also occurs in non-insulin-dependent diabetes mellitus (NIDDM) and that it lowers SUA levels by increasing the renal clearance of urate during the hyperfiltration phase[50]. They suggested that hypouricemia can predict the future progression of incipient nephropathy in NIDDM[50]. However, other reports have implied that high (and not low) SUA levels define the prognosis of chronic kidney disease (CKD)[51]. SUA is also associated with known risk factors for kidney disease progression[52], including hypertension[53], cardiovascular disease[54-56], and atherosclerosis[55]. SUA is an independent risk factor for CKD, even without diabetes[57].

SUA is known to be associated with disease progression in the early stage of diabetic nephropathy[17,58]. We found that the progression of renal dysfunction in patients with type 2 diabetic overt nephropathy with an SUA concentration of $\geq$ 6.3 mg/dL carries a poor prognosis, even though their SUA range is considered high-normal[59]. Our data shows the association between UA and disease progression is independent of diabetic control in multivariate analysis. Another report provided evidence for a clear dose-response relationship between SUA levels and early glomerular filtration rate (GFR) loss in patients with T1DM. The progression and regression of urinary albumin excretion were not associated with UA levels[60]. These studies show that UA is an in-

dependent risk factor for renal dysfunction, even after adjustments for confounding factors. Furthermore, even high-normal SUA levels accelerated renal dysfunction in T2DM patients[17,59-62].

UA is lowered in diabetes mellitus (DM) due to hyperfiltration[50], but decreased UA excretion during renal dysfunction raises SUA levels. Our previous study showed that UA levels in the patients who doubled Cr in the observation period (Cr doubling group) were higher than in the non-doubling group at the same estimated GFR (eGFR) level, suggesting that UA production was increased in the Cr doubling group[59]. These data suggest that higher levels of UA production are involved in the pathophysiology of nephropathy progression.

Several recent studies have been investigating therapeutic interventions to delay nephropathy progression[63-65]. Allopurinol therapy significantly decreases SUA levels in hyperuricemic patients with mild to moderate CKD. Its use is safe and has been shown to help preserve kidney function when used for a duration of 12 mo[63]. Febuxostat has a higher renoprotective effect than allopurinol, inhibits oxidative stress, has anti-atherogenic activity, reduces blood pressure, and decreases pulse wave velocity and left ventricular mass index, most likely due to a strong SUA lowering effect[65]. In an animal diabetic nephropathy model, allopurinol attenuated transforming growth factor-beta1-induced Smad pathway activation in tubular cells[66].

Diabetic foot

There are a few reports regarding the relationship between diabetic foot and UA levels. One study states that elevated UA levels are a significant and independent risk factor for diabetic foot ulcer in female Chinese patients with T2DM[67].

Macrovascular complication

A relationship between SUA levels and the development of atherosclerotic disease has been suggested[68-70]. Moreover, there is epidemiological evidence of an association between hyperuricemia and mortality in patients undergoing percutaneous coronary intervention or presenting with acute myocardial infarction[71-73]. Our study showed that SUA is an independent risk factor for vascular complications, even when adjusted for several confounders, including eGFR[56].

Macroangiopathy includes stroke, peripheral artery disease, and ischemic heart disease. In stroke, SUA levels are higher in patients with cardiac syndrome X, and elevated SUA levels are associated with carotid atherosclerosis[74]. A U-shaped relationship was shown for this correlation, as both the upper and bottom quintiles of SUA were associated with a higher risk of fatal stroke[75]. Besides, our study, a link between peripheral artery disease and UA has been rarely reported[56].

Several interventional studies have proven the efficacy of hyperuricemia treatments. A randomized controlled study showed that allopurinol prolongs exercise capacity (especially exercise time until ST depression) when a high dose of 600 mg/d of allopurinol was administered to patients with chronic stable angina[76]. Allopurinol treatment also protects the heart from ischemic reperfusion[77], and oxypurinol, an allopurinol derivative, improves the left ventricular ejection fraction (LVEF) in congestive heart failure patients with low LVEF[22]. Despite the numerous aforementioned studies, several studies have indicated that no association between UA and ischemic stroke[78] or heart disease[79] exists.

OXIDATIVE STRESS, ISCHEMIA/REPERFUSION, AND VASCULAR ENDOTHELIAL XDH/XO

UA itself reportedly functions as an anti-oxidant[80]. For example, XDH-null mutant Drosophila melanogaster have increased vulnerability to oxidative stress[81]. Uric acid administration improved endothelial function in the forearm vascular bed of patients with type 1 diabetes and smokers[82]. However, UA synthesis is accompanied by the generation of ROS.

XDH/XO in the vascular endothelium is associated with ischemia reperfusion injury. It has also been suggested that XO inhibitors improve endothelium-dependent vascular relaxation in blood vessels of hyperlipidemic rabbits[83]. XO as the source of ROS in ischemia/reperfusion injury has been discovered 30 years ago[84,85], and this injury is preventable with XO inhibitors[86]. XOR inhibition reverses endothelial dysfunction in heavy smokers[87,88]. XO inhibitors have the potential to act as free radical scavengers. Febuxostat, however, does not have this activity but can improve organ changes induced by ischemia/reperfusion[23].

FAT DIFFERENTIATION, INSULIN RESISTANCE, AND XDH/XO IN FAT CELLS

Adipose tissue has a high xanthine oxidoreductase activity in mice[1], and UA is secreted from adipocytes. XDH/XO is a novel regulator of adipogenesis and peroxisome proliferator-activated receptor gamma (PPARγ) activity and is essential for the regulation of fat accretion[89]. In addition, UA and adipose tissue XOR mRNAs are increased in ob/ob mice, and fat mass is reduced by 50% in $XOR^{-/-}$ mice.

ATHEROSCLEROSIS AND XDH/XO IN MONOCYTES/MACROPHAGES

XDH/XO is localized to CD68 positive macrophages in the pathological state[36,90]. Inhibition of XDH/XO in inflammatory mononuclear phagocytes inhibits the migration of neutrophils during acute lung injury[91]. Through inhibition of XDH/XO activity, cytokine-induced neu-

trophil chemoattractant secretion from mononuclear phagocytes is reduced, and small ubiquitin-like modifier of PPARγ and hypoxia-inducible factor 1α levels are increased[92]. Febuxostat activates mitogen-activated protein kinase phosphatase-1 and inhibits inflammation by lipopolysaccharide stimulation through the inhibition of ROS generation[93]. Tungsten, acting as a xanthine oxidase inhibitor, prevents the development of atherosclerosis in ApoE knockout mice fed a Western-type diet[94].

XDH/XO activity is also important for lipid accumulation[36]. XDH/XO knockdown or allopurinol administration inhibited foam cell formation in macrophage J774.1 cells. The production of inflammatory cytokines associated with foam cell formation was reduced by allopurinol and febuxostat, and these medications also significantly improved calcification and lipid accumulation in the aortic plaque of ApoE-KO mice[36,95]. It should be noted that the expression of XDH/XO and the deposition of UA are seen in macrophages in arteriosclerotic lesions[96]. *In vitro*, febuxostat inhibited cholesterol crystal-induced ROS formation[95].

Some reports describe XDH/XO as an endogenous regulator of cyclooxygenase (Cox)-2[97] in the inflammatory system, and XDH/XO is central to innate immune function[98]. XDH/XO is thought to be upstream of PPARγ in lipid retention[89] and also induces Cox-2 to induce inflammation, forming a potential feedback loop. In our study, administration of allopurinol to J774.1 cells inhibited secretion of inflammatory cytokines such as tumor necrosis factor α, interleukin (IL)-1β, and IL-6[36]. Gout-associated uric acid crystals activate the NALP3 inflammasome[99]. UA crystals can injure organelle such as lysosomes, and damaged organelle selectively sequestered by autophagy[100]. If mitochondria is damaged, autophagosome is driven *via* microtubule to NLRP3 inflammasome[101]. Colchine treatment expresses the anti-inflammatory effect for gout by inhibiting microtubule-driven spatial arrangement, not by inhibiting UA crystallization. Therefore uric acid crystal in inflammatory cells of atherosclerosis lesion might activate inflammation, while solvent uric acid acts as antioxidant. Microtubule-driven spatial arrangement might be a possible target for diabetic complication derived from UA crystals.

SIGNIFICANCE OF FUTURE UA METABOLISM RESEARCH FOR THE TREATMENT OF PATIENTS WITH DIABETES

XDH/XO has been studied for more than a century, and allopurinol has been used before enzyme inhibition therapy was established. In recent years, the various roles of XDH/XO in diverse pathological conditions have been revealed using a wide variety of research techniques, particularly in the field of molecular biology. This progress in research is related to the global demand to target lifestyle-related diseases such as T2DM, coronary artery disease, CKD, and MetS. Novel research has also led to the development of new powerful and safe UA lowering agent.

Obesity rates are increasing rapidly, and consequently, the pathophysiology of T2DM will be increasingly correlated with fat accumulation, chronic inflammation, and oxidative stress. UA metabolism (involving XDH/XO) is thought to play a central role in the pathogenesis of these conditions. Hence, the need for novel research will increase in the future.

CONCLUSION

The incidence of hyperuricemia has been on the increase since decades. The condition seems to be associated with increased insulin resistance and onset and progression of diabetic complications. UA might thus be suitable marker for both risk evaluation and intervention.

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REVIEW

Psychological aspects of diabetes care: Effecting behavioral change in patients

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Abstract

Patients with diabetes mellitus (DM) need psychological support throughout their life span from the time of diagnosis. The psychological make-up of the patients with DM play a central role in self-management behaviors. Without patient's adherence to the effective therapies, there would be persistent sub-optimal control of diseases, increase diabetes-related complications, causing deterioration in quality of life, resulting in increased healthcare utilization and burden on healthcare systems. However, provision of psychosocial support is generally inadequate due to its challenging nature of needs and demands on the healthcare systems. This review article examines patient's psychological aspects in general, elaborates in particular about emotion effects on health, and emotion in relation to other psychological domains such as cognition, self-regulation, self-efficacy and behavior. Some descriptions are also provided on willpower, resilience, illness perception and proactive coping in relating execution of new behaviors, coping with future-oriented thinking and influences of illness perception on health-related behaviors. These psychological aspects are further discussed in relation to DM and interventions for patients with DM. Equipped with the understanding of the pertinent nature of psychology in patients with DM; and knowing the links between the psychological disorders, inflammation and cardiovascular outcomes would hopefully encourages healthcare professionals in giving due attention to the psychological needs of patients with DM.

Key words: Psychology; Psychosocial aspects; Emotions; Cognition; Distress; Depression; Psychological resilience; Self-care; Coping behaviors; Quality of life; Diabetes mellitus

Core tip: Positive psychological health may sustain long-term coping efforts and protect patients from the negative consequences of prolonged emotional disorders, illness perception and thus facilitating diabetes self-management behaviors and better physical health. Having patients acquire valued personal beliefs and achievable standards of performance could strengthen self-regulation and self-efficacy leading to more positive experience and healthy behaviors. Furthermore, improved personal resources such as resilience would lead to better functioning of cognition and stronger will power, quality of life and disease control in patients with diabetes mellitus.

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INTRODUCTION

It is widely known that patients with diabetes mellitus

(DM) are at high risk of decreased psychological well-being[1-6] which is already presence in about half of the patients at the time of diagnosis[7]. This is due to strained coping with changed life routine (such as relationships, work-related and financial issues)[6] right from the time of diagnosis of DM[7]. An international survey, the Diabetes Attitudes, Wishes and Needs second study (DAWN2), included over 16000 individuals (comprising patients, family members and healthcare providers) in 17 countries across four continents, reported that the proportion of the people with DM who were likely to have depression and diabetes-related distress (DRD) was 13.8% and 44.6%, respectively, with overall poor quality of life at 12.2%[8].

DM had a negative impact on many aspects of life, ranging from 20.5% on relationship with family or friends to 62.2% on physical health. About 40% (18.6%-64.9%) of these patients reported their medication interfered with their ability to live a normal life[8]. Furthermore, these patients often use negative coping strategies and more frequently perceive that diabetes would negatively affect their future[4,7]. Untreated psychosocial disorders in DM, may lead to more physical symptoms[9], cardiovascular complications[10] and depression[11,12]. Depression may lead to cognitive decline and further aggravate the vicious cycles of self-care ability[13]. Many previous studies have largely been on the relationship between depression and diabetes[14,15], with the focus on major depressive disorder. However, sub-syndromal depressive and milder emotional conditions, such as dysthymia, anxiety, stress and distress[16], are far more prevalent than major depressive disorder especially at the primary or community care levels[17,18]. Furthermore, these emotional disorders are linked to increased disability, risk of health decline, healthcare use and premature mortality[17,19,20]. Despite the widespread prevalence of psychological problems and their negative consequences, the availability of person-centered chronic illness care and psychological support was low for patients with DM. Only 48.8% had received psychological treatment or educational activities to help manage their diabetes[8]. This review discusses patients' psychological aspects in general with a focus on emotion effects on health, and emotion in relation to other psychological domains such as cognition, resilience, willpower, self-efficacy and behavior. Furthermore, this review reports recent findings on the links between psychological disorders, inflammation and cardiovascular outcomes in patients with DM.

Equipped with the understanding of the pertinent nature and impacts of psychology in patients with DM, it is hoped that this review would encourage healthcare professionals in giving due attention to the psychological needs of patients with DM.

RESEARCH

We conducted searches of multiple databases [MEDLINE® *via* PubMed®, Embase®, Cochrane Register of Controlled trials, CINAHL (EBSCO), PsycINFO] using terms for emotion, cognition, human behavior, psychosocial and psychological aspects in diabetes care, including but not limited to MeSH terms for emotional disorders, depression, anxiety, stress, distress, diabetes mellitus and psychological interventions. We obtained additional articles from systematic reviews; reference lists of pertinent studies and editorials. We compiled a narrative synthesis of findings, highlighting underlying theories, mechanisms and interactions of the different and essential psychological aspects of patients that might influence self-care behaviors and clinical outcomes.

HEALTH EFFECTS OF EMOTIONS?

Under-expression or over-regulation of emotions with all the other dysfunctional control of emotions could be both the causes for and results of inappropriate emotional responses, personality or even psychiatric disorders[21,22]. These have been inevitably shown to be associated with physical health[11,23,24] and DM[25].

Conversely but in parallel to previous observations, Pressman and Cohen proposed links between positive affect or emotions and health[26]. They suggest that emotion has a direct effect on both behavior and physiology. More specifically, they hypothesized that positive emotions, such as happiness, excitement and contentment result in better health behaviors and improved adherence to treatment regimens. Direct physiological effects include autonomic nervous system activation, hypothalamic-pituitary-adrenal axis activation (decreased cortisol), and on immune functioning through the primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid tissues[27,28]. Indeed, some evidence exists for a moderating effect of emotions on natural killer cell activity[29]. In a 20-year follow-up study[30], baseline feeling of vigorous at work among the healthy employees had lower risk of mortality (HR = 0.74, 95%CI: 0.58-0.95) and incidence of diabetes (HR = 0.83, 95%CI: 0.68-0.98) after adjusting for the total cholesterol, glucose, body mass index, smoking, alcohol intake, physical activity, depressive and anxiety symptoms. Healthy behavior such as physical activity causes endorphin excretion leading to a sense of elation[31], which further reinforces the behavior through operant conditioning. It appears then that as if there is a "spiraling up" of positive effects from physical and psychological being within a person in contrast to the opposite "vicious cycle" of negative emotions.

The pathways between negative and positive emotions and health outcomes interact through behavioral and/or biological mediators, both of which have relevance for DM, an illness characterized by underlying inflammatory changes[32,33]. Negative emotions can intensify a variety of health threats. Stress, anxiety and depression are related to impaired immune, pro-inflammatory cytokines and inflammation responses that have been linked to a spectrum of conditions associated with aging, including cardiovascular diseases, osteoporosis, arthritis,

Alzheimer's disease, frailty and functional decline, DM, certain cancers and periodontal diseases[24,34]. Additionally, negative emotions could contribute to prolonged infections and delayed wound healing, conditions that further enhance pro-inflammatory cytokine production[24]. Accordingly, distress-related immune dysregulation may be the underlying mechanism of a larger and diverse set of health risks associated with negative emotions. Thus, the relationship between emotional disorders and inflammatory responses is likely to be synergistic and bi-directional- the vicious cycle effect[34].

WHAT IS EMOTION?

An overarching aspect of theoretical perspectives represented in the past three decades of research is that emotion and cognition, though often perceived as having separate functional features and influences[35,36], are indeed highly interactive and integrated in the brain[37-39]. This notion is consistent with the high degree of connectivity within the brain's neural structures and systems. Therefore, emotion is hypothesized to have substantial and measurable effects on cognition and action (behavior) when the stimulus or situation is personally or socially significant to the person involved[37,40]. The key principle of differential emotions theory states that emotions play central role in consciousness and awareness, having dynamic neurobiological and neuropsychological activities that lead to continuous emotions-cognitions interaction in influencing adaptive thoughts and actions as manifested in decision making and behavior[40].

Physiologically, emotion constitutes brain responses and body expressions[41]. Although there is no consensus on a general definition of the term "emotion"[42], many experts do agree that emotions have a limited set of components and characteristics. In addition, emotions have an infrastructure that includes neural systems dedicated in parts to emotion processes and recruit response systems when emotions motivate cognition and action. The autonomic nervous system modulates the intensity of the emotions but does not change its quality or valence. Feeling is a component of emotion that is always experienced or felt, though not necessarily labeled or articulated or present in access consciousness (a level of consciousness that has reportable content). It is considered to be a phase (not a consequence) of neurobiological activity that is sensed by the organism[40] and was reported to be present and expressed even in children without a cerebral cortex[43]. Current evidence suggests that in goal-oriented behaviors, the feeling component of emotions contribute its effect to the evolution of consciousness, cognition and action processes resulting in the behaviors[40].

There is a consensus that emotions exist in different forms: (1) basic emotions, those that are probably universal and involve less cognitive complexity for example anger and fearfulness, appear primarily in evolution and biology; and (2) emotion schemas, that include cognitive components differ across individuals and cultures[44,45]. Basic emotions usually occur in acute situations and easily bypassing cognitive process in favor of a quick reaction to the situations. Emotion schemas are emotions that have been interpreted by the cognition.

Past experience and emotion

Experience is emotional historical facts, similar perhaps to a textbook of history that is none other than a compilation of factual events. Without emotions, every life experience would be reduced to none others but a talking history textbook. There are no memories without emotions just as there are no persons without experience. Past experience becomes memory because of the emotional content it carries. Accumulated past experience influences personality and personal belief systems in an individual[46], and shapes the cultural behaviors in the family and community[47]. The flavor of these memories depends on personal interpretation of the meanings of the experience. Although the objective events would arouse universally similar emotions, its unique interpretation will lead to different meanings for the person experiencing them. This is where the effect and influence of cognition comes in. Thus, emotions serve like a repository for learned influences, possessing certain invariant features and show considerable variation across individuals, groups, and cultures[48].

These past experiences, crystalized as emotions, facilitates learning and motivates preparedness for future interactions with people, events, and situations. Evidence indicates that experimentally facilitated formation of emotion-cognition interaction i.e. schemas (such as simply learning to label and communicate about feelings) generates adaptive advantages[49,50]. The dynamic interplay of emotion and cognition determines many human behaviors, for example connecting appropriate cognition to feelings increases the individual's capacity for emotion modulation and self-regulation[49]. The first step towards initiation of action is by improving the perception of emotions that entails the registration of emotions in the consciousness. This is made possible by the ability to symbolize feelings and put them into words thus providing an empowerment for emotion regulation, influencing emotion-cognition relations and developing high-level social skills. Without this, the unlabeled, unarticulated, and linguistically inaccessible emotional feelings would be in the phenomenal consciousness or some other cognitively inaccessible level of consciousness although it can still be felt and functions as a mediator of behavior, retaining its motivational and informational qualities[49].

EMOTION AND COGNITION

Emotion alone could never be the sole mediator of personally or socially significant behaviors. Other persons and contextual variables do also contribute to the causal processes of certain behaviors. However, it is proposed that emotion is always one of the mediators of a behavioral action in response to basic emotion and a mediator

of thought and action in response to emotion schemas[40]. Therefore, the specific impact of emotions in generating and altering behavior depends on the type of emotion involved in the causal process. In basic emotions, feelings affect action but not higher-order cognition, which has little influences in the basic emotion processes. In contrast, feeling in emotion schemas may frequently effect action through its effect on the cognition. Hence, thinking becomes a key agent in regulating and guiding behavior that arises from the emotion schemas[51].

A cognitive appreciation of emotions in relation to the issue or event at hand turns out to be the actual initiator of decision-making. In other words, a person agrees to do an action because he or she feels right and happy about the intended action, and apply controlling power over or drawing its motivation from the emotions. The direction of this decision could be at its best instinctive (without cognitive appreciation-the basic emotions[44]) and primitive (the emotion schema)[44]. if it is not based on and guided by higher moral value. This higher value system is closely related to the concept of purpose in life in many resilience studies[52-54]. This higher value could arise from the self-generated value system (close-system) or be imparted from the supreme beings or religion-based value system (open-system)[55]. These three tiers of the action-sources in the interplay of the emotion-cognition-higher value system could distinguish between hot (impulsive), cold (ordinary) and extra-ordinary men, respectively.

EMOTION, COGNITION AND BEHAVIOR

The current perception is that emotion remains primarily about motivation[56], while cognition (particularly about goal concepts that typically have an emotive component) remains primarily about knowledge. The presence of both is almost always the case in any normal human being for his or her normal social functioning[57]. However, they could differ in sequence of activation and intensity depending on the stage of life and situations the person is in[57,58]. The presence of both the emotion and cognition is invariably necessary for adoption of new life skills and adaptation to new environments[59].

Emotional intensity theory suggests that emotions have motivational properties because they furnish energy and direction for the execution of appropriate instrumental behaviors[60,61]. Specifically, emotions promote fast adaptation to situational demands by helping individuals to identify relevant and important events and by urging, guiding, and maintaining the behaviors necessary for dealing with these events[48,60]. For instance, if someone is insulted and experiences anger, all biological systems and resources are coordinated so that the person can deal efficiently with the situation while ignoring all other signals and events. Thus, affective systems are designed to conserve energy and mobilize resources to achieve a short-term goal. These emotions are typically short-lived psychological-physiological phenomena that represent efficient adaptations to the demands of the changing environments. Psychologically, emotions activate relevant associative networks in memory, which alter attention and shift certain behaviors upward in the response hierarchies. Physiologically, emotions rapidly excite and orchestrate the responses of various biological systems, including the autonomic nervous system activity and endocrine activity, to produce a bodily milieu that is optimal for effective response. The manifestations include facial expression, somatic muscular tonus and voice tone. Therefore, over longer periods of time, with many of these emotional encounters, people mature through the ages[62], emotion-enriched experiences serve to establish our position in our environment, drawing us toward certain people, situations, objects, actions and ideas, and pushing us away from the others.

Because emotions are viewed as motivational states, their intensity should be effected by factors similar to those influencing the intensity of regular motivational states[61]. Events that interfere with the experience of an emotion can influence the intensity of that emotion. Past work has shown that emotional intensity was similar to motivational arousal, which could be jointly influenced by the importance of a goal and the difficulty of achieving it[61]. In the case of anger, events that interfere with feeling or expressing anger can affect its intensity.

The interaction between emotions and cognition in decision-makings has also been reported where emotion, in particular worry, has been shown to cause more short-term decision (cognition domain) over long-term choices that may have significant consequences to health[63]. Emotional regulation *via* cognition such as cognitive re-appraisal and expressive suppression are shown to lead to better social adjustment, mental health and overall well-being[64]. Furthermore, cognitive training in patients with psychiatric disorders (schizophrenia, attention deficit hyperactivity disorder, mood disorders and substance use disorders) could improve emotional regulation, clinical symptoms, and adaptive community functioning[65]. This concept of emotional regulation as related to willpower elaborated below is invariably associated with physical health too.

Self-regulation

Self-regulation has its major explanatory mechanism in social cognitive theory[66]. Self-regulation that is effective results in execution of a behavior and suppression of another competing but undesirable behavior. It begins from having a valued personal standard on certain actions or behaviors, which would then generate heightened motivation in realizing the action-behavior. Execution of certain actions or new behaviors is sometime aided by proactive consideration of the possible effect or consequence of the current actions-behaviors in the future, or evaluative reactions of others towards one's behavior. Self-monitoring of performance would compare the outcomes of the performance to social or personal past referential achievement[66]. Without comparison to

the valued extrinsic outcomes, there would be absence of meaningful feedback that could in turn activate self-evaluative motivators.

Psychological functions described by self-regulation include components of self-discipline, self-reactive influences and self-gratification[66]. It is presumed that the common values or motives within every individual are beneficial, self-constructive, pro-social and respectable. There are no objective universal referent standards that every individual could subscribe to besides those that are subjective and internal within that individual, and those that are external on the society or significant others at large. This socio-cognitive functioning of self-regulation in decision making for or against certain action learn from past experience of exercising control over the dynamic environment. Through this repeated process, conceptual skills become acquired skills and self-efficacious[66].

Overt self-centeredness of this theory predisposes to self-love at best and despondency or depression at worst from dysfunctional self-regulation as a result from misperception on performance standards and misjudgments on achievement of self[66,67]. It is a closed system that could suffer from inconsistency of the internal standards as compared to the more universal moral standards[68]. As a result, it would also suffer from a sense of helplessness and hopelessness[69] from devoid of the ultimate source (supreme beings or God in the open-value system) of help and hope in the face of weakened coping efficacy and beliefs which is highly possible in many chronic diseases self-care failures such as in patients with DM. This external source of the internal reserve may enable a self-renewal for a new beginning of coping with life challenges. Hence, it is not impossible that religiosity and spirituality could affect glycemic control[70].

Self-efficacy

Self-efficacy is embedded within the theory of self-regulation[66]. It operates as one of the main proximal determinants of self-regulation though self-monitoring, goal setting and valuation of activity sub-functions. Self-efficacy is self-confidence or self-believe in one's own ability to carry out or overcome difficulties inherent in specific tasks[71]. Hence, beliefs of one's own efficacy cause people to make choices, aspire and persevere in things that they have the confidence in achieving. This theory suggests that people with higher self-efficacy would keep improving in life due to their positive self-feedback and setting higher new targets to achieve in progressive efforts.

This confidence stems from learned capability gained through past experiences when efforts were expended for the behaviors[72]. In this theory, differential experience and cognitive processing of efficacy information lead to different degree of self-efficacy attainments. The intervening link between the efficacy expectation and the actualization of efficacy in action could be self-aiding thoughts, the emotion-motivation fortified resilience that is powered by the activated personal value or belief system. However, similar to its parent theory of self-regulation, self-efficacy theory relies too heavily on self-centeredness, autonomous judgments and could result in both extreme ends of self-destruction, *i.e.*, over-confidence and self-despair.

Willpower

Willpower functions like an "actualizer" of the formed intentions into real behaviors[73]. It employs conscious and effortful self-control when faced with life choices or temptation and manifests as an ability to resist short-term gratification for long-term return[74]. With willpower, people overcome "hot" emotional pushes with the "cool" cognitive capacity[73]. Thus, willpower is an educated spirit that grows on understanding and has the ability to control emotions. Willpower is likened to a trait as evidenced by studies demonstrating that the similar quality of the willpower that appeared in the preschoolers persisted into adulthood[75,76]. Past studies show that willpower was positively correlated with many aspects of life such as better academic achievement in schools, higher self-esteem, lower substance abuse rates, greater financial security and improved physical and mental health[75,77].

The effects of willpower could however deplete if it is repeatedly exerted within a short span of time and thus is predisposed to failure of self-control in an immediate next challenge[78]. Thus, willpower depletion is best avoided by focusing on one task at a time as it has been observed that willpower fares optimally when it is applied on one valued goal after another instead of multiple resolutions at once[79]. This will negate the impact of willpower failure on a range of potential challenging behaviors such as food intake, substance use and abuse and purchasing behavior[80-82]. Elsewhere it has been shown that people with positive moods, motivation, beliefs and attitudes or vitality were found to be more able to mitigate this depletion and to persevere even when their willpower strength has been depleted[83-85]. Thus, positive emotions bolster willpower when it is weak but negative emotions, on the other hand, could be suppressed by the willpower when it is cognizant in according to the situations. Interestingly, it was noted that willpower resembled resilience in that regular exertion of self-control improved willpower strength over time[86].

Resilience

Resilience is defined as an individual's capacity to maintain psychological and physical well-being when faced with adverse life events by drawing on self-esteem, self-efficacy, self-mastery and optimism as resources[52-54]. Other qualities of resilience include internal locus of control, social support and purpose in life[87]. These personal qualities vary among different individuals depending on whether the events are perceived as stressful, a threat or a challenge[88]. Resilience has been shown to contribute to relatively successful social functioning in the elderly with DM, with an effect that was stronger than social support and material resources[89].

It has often been a phenomenon that adversity breeds

resilience as in the analogy of a well rooted strong tree growing up in the wilderness. In man, brief and graded exposures to stressors in turn would allow cumulative experience, learning and strengthening of a person (the steeling effect)[90-92]. Thus, there is no true resilience in the absence of true adversity[90]. External adversity makes assessment of resilience comparable across individuals. Hence, subjective interpretation of internal adversity (such as in sickness) is acceptable as the adversity is being faced by an individual with his or her own unique socio-biology milieu.

Behaving resiliently is only possible if there are reserves and resources to draw from. Reserves are internal strength of the person which when tested in the face of adversities, could either manifests in positive emotions (hope, optimism, happiness and vitality) or in negative emotions (apathetic, feel guilty, overwhelmed, disgruntled and depressed). Resources are external supports of all possible forms from every potential party. Between these two, reserves would be a closer and stronger resilient factor for simply being a more personal characteristic in the face of almost all adversity because no adversity is an adversity if it does not affect at the personal level and demand a personal response. This internal reserve depends largely on the personal value and belief system that could result from the past experience (emotional learning), educated cognition (knowledge) or relationship with a supreme being(s)[55,87,88]. The inter-play and effectiveness of each of these factors would have manifestations that mirror the three tiers of human-action or behaviors namely; the beast-like reflex action, the ordinary but superficial culture and politeness; and extra-ordinary self-sacrificial altruism. The great divide between these factors would be the self-dependency in the former two and depending on the supreme-value or being God-dependent in the last. This divide is not necessarily mutually -exclusive but perhaps reflective of a responsible, balanced and appropriate execution of dependency on self and supreme beings or God. The greatest danger of self-dependency is probably self-deception resulting from misperceptions and self-isolation; while supreme-value or God-dependency could be far reaching for the majority, as the supreme beings/God are/is too abstract to be real as in the demand of religious faith[55].

Illness perception

Illness perceptions involve beliefs, cognitive and emotional representations or understandings that patients have about their illness[93]. These perceptions have been found to be associated with health behaviors and clinical outcomes, such as treatment adherence and functional recovery[94]. Illness perceptions constitute beliefs on the chronicity of the illness, locus of control of the illness and efficacy of treatments; it includes an assessment on the perception of understanding the patient has of the illness; illness perception evaluates the emotional impact of the illness directly and indirectly from the aspects of symptoms experience and concern for the illness's consequences.

Some of these illness perception dimensions had small significant associations with HbA1c[95]. Tentative evidence indicate that illness perceptions can be positively changed through targeted intervention and that could have an impact on glycemic control[95]. Patients' perception of their illnesses and related symptoms and their beliefs about the possible consequences of the disease had also been shown to be associated with their satisfaction with medical consultation and healthcare utilization, respectively[96]. Misperception could complicate reassurance[96] from healthcare professionals and impede self-coping on patient's part[94].

Proactive coping

Future-oriented thinking or the proactive coping concept goes a step further in explaining how people could maintain an acquired behavior[97]. In this model, a person who practices proactive coping is said to be in continual anticipation of the potential barriers and threats to the lapses of the desired behavior; have the ability to develop and realize the strategy to offset the threats. In addition to the effective use of resources, the person who is successful in maintaining his or her behavior would also use effective feedback on self-strategy to keep the goals viable. In a study of newly-diagnosed DM patients, proactive coping was shown to be a better predictor of long-term (at 12 mo) self-management (diet and physical activity and weight loss) than either intentions or self-efficacy[98].

However, it is proactive coping rather than future-oriented thinking that seems to be more feasible and in line with other health behavior concepts. Knowing the immense possibilities of the distant future and demands of the present in self-management coping for DM might overwhelm the emotion and crumble the present functioning of a person. Applying proactive coping even for near proximal outcomes may require high degree of support, emotional and cognitive agility to succeed[99]. Hence, patients with adequate cognitive and emotional resource and reserve would likely to cope proactively[100]. Issues remain in individualization of such behavior, matching its intensity to the patient's characteristics and valued goals in life in order to preserve acceptable level of quality of life. Therefore, patients who can behave and cope proactively are those who have a right illness perception (right understanding about DM), perceive its importance in their life, have self-efficacy and able to self-regulate.

NEGATIVE PSYCHOLOGICAL EFFECTS ON DIABETES MELLITUS

In adults, children and adolescents with DM, depression was related to poorer glycemic control, a range of diabetes complications, increased health care costs, worsened functional disability, re-hospitalization and early mortality[101]. Those with psychological distress at the time of diagnosis had a higher risk of cardiovascular events

(1.7-fold) and death (1.8-fold) than those without psychological distress[102].

Emotions and the brain in DM

Current research suggest biological changes in the brain of patients with DM. Structural, functional, and neurochemical changes in the brain regions responsible for affect and cognition may have increased the risk of depression in both type 1 and type 2 DM[103]. Animal models have shown that hyperglycemia negatively affect hippocampal integrity and neurogenesis, reducing neuroplasticity and contributing to mood symptoms[104]. In humans, hippocampal neurogenesis and hippocampal atrophy has been observed in people with DM, which will lead to difficulty in learning, maintaining memory and governing emotional expression[104].

Emotions and systemic inflammation in DM

In a recent published study in United Kingdom[10], depressive symptoms in adults with newly diagnosed type 2 DM, after adjusting for covariates, were associated with systemic inflammatory markers: C-reactive protein (B = 0.13, $P < 0.001$), interleukin-1β (B = 0.06, $P = 0.047$), interleukin-1RA (B = 0.13, $P < 0.001$), monocyte chemotactic protein-1 (B = 0.11, $P = 0.001$), white blood cell count (B = 0.13, $P < 0.001$), and triglyceride (B = 0.10, $P < 0.001$).

The effect of negative affect and moods on the inflammatory markers, immune systems and endothelial functions are further compounded in patients with DM[105]. This is because hyperglycemia in diabetes has already deleterious effect on the endothelium[106,107]. The "glucose tetrad" of HbA1c, glycemic variability, fasting and postprandial plasma glucose activate oxidative stress causing vascular complications through endothelial dysfunction and damage[108]. Chronic glycation of mitochondrial respiratory proteins leads to mitochondrial DNA damage and functional decline causing over-production of intracellular free radicals and perpetual cellular injury[109]. Non-enzymatic glycosylation of other proteins and lipids by disrupting their molecular conformation alter many enzymatic activities, reduce degradative capacity and interfere with receptors recognition[110]. The presence of hypertension and hyperlipidemia in patients with diabetes impose added detrimental effect on the micro- and macrovasculature. These include cholesterol oxidation and glycosylation contribute to the progression of atherosclerosis by promoting vascular smooth muscle cells migration and proliferation[111]. In the hypertensive diabetes patients, impaired auto-regulation in the microcirculation with non-dipping of nocturnal blood pressure leading increased pulse-wave velocity, ventricular-vascular mis-coupling and premature stiffening of the abdominal aorta owing to autonomic dysfunction and elastic fibres glycation[112].

Emotion lability and biomarkers variability

It is widely observed that emotions are relatively stable over time, constitute the person general outlook and represent personality. However, it is possible that affects change from time to time. It was reported that changes in affects and emotions over a short period of time were detrimental to health, especially in the cardiovascular organ systems through the sudden or unpredictable surge in pulse rate and blood pressure[22,113]. Dysregulation of emotions can impact on physical health through the autonomic nervous system activation and hypothalamic-pituitary-adrenal axis activation that affect the metabolic and immune functioning of a person[11,23,24,27,28]. Therefore, it is hypothesized that unregulated emotional fluctuation could lead to variability in blood pressure and glycemic control biomarkers. In the reverse direction, Penckofer[114] had reported that glycemic variability measures were associated with mood (depression, trait anxiety and anger) and quality of life. The 24-h SD of the glucose readings and the continuous overall net glycemic action measures were significantly associated with health-related quality of life (HRQOL) after adjusting for age and weight; and subjects with higher trait anxiety tended to have steeper glucose excursions.

In patients with DM, a recent Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation trial had reported clear associations between visit-to-visit variability (VVV) of HbA1c and the risk of macrovascular events ($P = 0.02$ for trend), whereas fasting glucose variability was associated with both macro- and microvascular events ($P = 0.005$ and $P < 0.001$ for trend, respectively)[115]. In an earlier study it has been shown that HbA1c variability affects nephropathy more than average HbA1c, whereas only the latter parameter affects retinopathy[116]. On the other hand, glucose variability as characterized by extreme glucose excursions, independent of HbA1c levels, could be a predictor of diabetic complications (development or progression of diabetic retinopathy and cardiovascular events) and mortality in patients with DM[117]. The mounting evidence on these associations suggest that increased frequency and magnitude of glycemic variability generates more reactive oxygen species that triggers the various metabolic pathways of glucose-mediated vascular damage which result in an increased risk for the development of long-term diabetic complications[118,119].

Similarly, VVV in systolic blood pressure (SBP) and maximum SBP are strong predictors of stroke, independent of mean SBP[120]. Increased residual variability in SBP in patients with treated hypertension was associated with a high risk of vascular events[120]. In each TIA cohort, VVV in SBP was a strong predictor of subsequent stroke (top-decile hazard ratio over seven visits: 6.22, 95%CI: 4.16-9.29, $P < 0.0001$). In ASCOT-BPLA[121], residual VVV in SBP on treatment was also a strong predictor of stroke and coronary events (top-decile HR for stroke: 3.25, 2.32-4.54, $P < 0.0001$), independent of mean SBP in clinic or on ambulatory blood pressure monitoring (ABPM). Variability on ABPM was a weaker predictor, but all measures of variability were most predictive in

younger patients and at lower (< median 142.8 mmHg) values of mean SBP in every cohort[120]. However, there is no evidence to date that suggest similar detrimental effects of cholesterol variability in adult patients with DM.

PSYCHOLOGICAL INTERVENTION IN DIABETES CARE

Despite evidence that psychosocial support was instrumental to adaptive self-care as indicated by patients in the DAWN2[6], psychosocial and pharmacologic interventions have not been widely used to target psychological co-morbidities such as depression and DRD[122]. The psychosocial supports through caring and compassionate family, friends, health care professionals, and even other patients with DM could instill a positive outlook, sense of resilience and wellbeing in patients with DM. Screening, evaluation and management of psychological disorders such as depression and DRD in people with DM in primary care are feasible[123].

Indeed, positive psychosocial factors are important mediators or independent predictors of clinical outcomes in chronic diabetes care and positively related to self-care behaviors[124]; exerting a direct impact on HRQOL and subjective health. A recent review[125] and study[126] reported that positive emotional health (well-being, positive affect, resilience and gratitude) were linked to self-management (exercise, treatment adherence and frequency of blood glucose monitoring), health-related outcomes (HbA1c, health status and HRQOL) and lower risk of all-cause mortality in patients with DM[3,125]. However, few quality studies have investigated the effects of positive aspects of emotional health (resilience, positive affect, well-being) on patient outcomes; even lesser empirical studies showed strong evidence of the actual effect of positive and negative affect on glycemic control[127,128]. Although the interaction between emotional health and diabetes physiology and patient's self-care practices that in turn further influence health outcomes are becoming clearer, there is still a paucity of health programs that incorporate human psychology wholesomely and intervene effectively in patients with DM for improved self-care behaviors and clinical outcomes[129,130]. Some recent studies that examined depressive symptoms and DRD and their management has found cross-sectional, prospective and time-concordant relationships with HbA1c[131,132]. Nevertheless, a causative relationship between the two requires more significant prospective linkages between DRD and HbA1c[132]. From the discussion above, it is possible that emotional disorders can affects HbA1c in a bidirectional pattern[133]; from distress or depression to DM *via* lifestyle factors and due to therapeutic demands in the reverse direction[133].

Notwithstanding, interesting questions emerge whether interventions involving psychological, intra- and interpersonal resources may be possible to buffer the negative inflammatory effects of emotional disorders in patients with increased risks of cardiovascular diseases such as in patients with DM. Improving cognitive appreciation in education, increasing positive affect and motivation to initiate positive lifestyles could in turn lead to better self-care behavior and quality of life. Therefore, interventions that focus on positive emotional health to diminish negative emotions could enhance health in part through their positive impact on immune and endocrine regulation, resilience, self-efficacy, positive behaviors and HRQOL[34].

The immediate next questions would be: (1) How much of these effects could be achieved in patients and within their family members? (2) How personalized should the interventions be? and (3) How much do the existing health systems need or able to transform in order to implement the interventions? These questions consider other potential social determinants of DM that may influence effectiveness in diabetes care provision[134]. The first question involves the essential issue of the characteristics of patients in participating the interventions for example their pre-intervention health beliefs and barriers to change assuming the interventions that follow would help them to put right most if not all health beliefs and behaviors. The second question involves having cost- and content-effective interventions[135,136] that may need to be separately prepared for patients at different stages of diseases for example newly diagnosed DM, persistent poor control of disease, impending or newly diagnosed complication/comorbid; or going into different life stages such as young working adults, family planning or pregnancy, retirement and above 60-year-old[137]. The personnel to deliver the interventions will need training that would enable them to conduct a flexible, dynamic and culturally appropriate interventions[136,138,139]. The third question implies staff and health system readjustment and investment to begin the intervention[140,141], to maintain and even to continuously update the interventions in accordance with the contemporary evidence of medicine[142]. The ultimate aims would be to help individual patient to develop own strategies for the long-term management of their diabetes, and that at the same time leading a productive life resulting from a quality of life that is resilient to adversities and challenges.

CONCLUSION

Understanding the nature of the psychological aspects that are pertinent in patients with DM, and the links between the emotional disorders (stress, distress, anxiety, DRD and depression) and inflammation has provided a mechanistic insight into the relationships between psychological domains and poor physical health[34]. Positive emotional health may sustain long-term coping efforts and protect patients from the negative consequences of prolonged emotional disorders[143], illness perception and thus facilitating diabetes self-management behaviors and better physical health. Having patients acquire valued personal beliefs and achievable standards of performance could strengthen self-regulation and self-efficacy and lead to more positive experience and healthy behaviors.

Furthermore, improved personal resources such as resilience would lead to better functioning of cognition and stronger willpower, quality of life and disease control in patients with DM. More research is needed to understand what factors contribute to individual DM differences in vulnerability, treatment response and resilience to psychological disorders and cardio-metabolic risk factors control across the life course. More international collaboration is helpful to examine how best to provide care for people with DM and emotional disorders in different health care and cultural settings. Psychological training programs grounded on sound theoretical framework such as that draw on the fundamental value system or personal purpose in life could effect powerful involvement of emotion and cognition leading to meaningful and lasting behavioral change. Lastly, a cross-disciplinary workforce is necessary and the program should be culturally flexible for it to work in different models of healthcare system and for patients with DM of different backgrounds[101].

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REVIEW

Association of genetic variants with diabetic nephropathy

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Abstract

Diabetic nephropathy accounts for the most serious microvascular complication of diabetes mellitus. It is suggested that the prevalence of diabetic nephropathy will continue to increase in future posing a major challenge to the healthcare system resulting in increased morbidity and mortality. It occurs as a result of interaction between both genetic and environmental factors in individuals with both type 1 and type 2 diabetes. Genetic susceptibility has been proposed as an important factor for the development and progression of diabetic nephropathy, and various research efforts are being executed worldwide to identify the susceptibility gene for diabetic nephropathy. Numerous single nucleotide polymorphisms have been found in various genes giving rise to various gene variants which have been found to play a major role in genetic susceptibility to diabetic nephropathy. The risk of developing diabetic nephropathy is increased several times by inheriting risk alleles at susceptibility loci of various genes like *ACE*, *IL*, *TNF-α*, *COL4A1*, *eNOS*, *SOD2*, *APOE*, *GLUT*, *etc*. The identification of these genetic variants at a biomarker level could thus, allow the detection of those individuals at high risk for diabetic nephropathy which could thus help in the treatment, diagnosis and early prevention of the disease. The present review discusses about the various gene variants found till date to be associated with diabetic nephropathy.

Key words: Diabetes mellitus; Diabetic nephropathy; Genetic polymorphism; Gene variants; Nephropathy

Core tip: Diabetic nephropathy is actually the most common cause of kidney failure. It is now a scientifically proven fact that there is a strong association between an individual's genetic makeup in his predisposition to diabetic nephropathy. Multiple genes are involved in pathogenesis of diabetic nephropathy, with several allelic polymorphisms having demonstrable effects in the development and progression of the disease thus contributing to the overall risk. These gene polymorphism studies are thus conducted to identify at-risk patients and design therapeutic strategies to prevent the outcome of such complication in his later future. This review discusses about the various gene variants found till date to be associated with diabetic nephropathy.

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INTRODUCTION

Diabetes mellitus is a complex syndrome leading to various metabolic dysfunctions. These metabolic dysfunctions manifest characteristic long-term complications in the form of various microvascular diseases, including diabetic nephropathy, retinopathy, and neuropathy. Diabetic nephropathy is one of the major secondary complications of diabetes mellitus affecting almost 40% of the diabetic patients. Diabetic nephropathy is clinically characterized by proteinuria, declining glomerular filtration rate, hypertension eventually leading to renal failure, requiring dialysis or transplantation. Various risk factors like, hyperglycemia, increased blood pressure, and genetic

alterations may predispose an individual to diabetic nephropathy in the near future[1]. It is now a scientifically proven fact that apart from the above risk factors, there is a strong association between an individual's genetic make-up in his predisposition to diabetic nephropathy. In this context, Andersen *et al*[2] have shown that 35% of the patients with diabetes develop nephropathy, irrespective of glycemic control. Identification of genetic components of diabetic nephropathy is the most important area of diabetes research because elucidation of genes (alleles) associated with diabetic nephropathy will influence all efforts toward an understanding of the disease at molecular and mechanistic levels, its related complications, cure, treatment and prevention. Association studies of candidate genes for diabetic nephropathy are being conducted all around the globe to identify the biomarkers genes which may predispose a diabetic individual to the risk of diabetic nephropathy. Among the genetic factors involved, single nucleotide polymorphisms in the genes associated with diabetic nephropathy was found to have a major impact on the disease outcome. These gene polymorphism studies are thus conducted to identify at-risk patients and design therapeutic strategies to prevent the outcome of such complication in his later future.

GENE VARIANTS ASSOCIATED WITH DIABETIC NEPHROPATHY

It is now a scientifically proven fact that genes are amongst the major contributors to diabetic nephropathy apart from the environmental factors involved. In this context, a wide range of genes have been assessed to see their association with diabetic nephropathy along with a number of single-nucleotide polymorphisms in diabetic nephropathy susceptibility genes[3]. It is seen that different ethnic groups may have variable risk associated with a specific gene in individuals suffering from a particular disease like diabetic nephropathy. Given below is a discussion of few genes involved with diabetic nephropathy.

Inflammatory cytokines gene variants

Inflammatory cytokines are involved in pathogenesis of diabetic nephropathy and the genetic variability in the genes encoding these cytokines may predispose a person to diabetic nephropathy. Some of the cytokine gene variants found to be associated with diabetic nephropathy are as below.

Interleukins: There is a significant association between carriage of interleukins (IL)-1β allele 2 (-511 C/T polymorphism) and IL-1RN (IL-1 receptor Antagonist gene) allele 2 (2 copies of the repeat sequence) with diabetic nephropathy. In case of *IL-6* gene, C/G polymorphism at position 634 in the promoter region of the *IL-6* gene is a susceptibility factor for the progression of diabetic nephropathy where G/G homozygote showed a significant positive association with macroalbuminuria in type 2 diabetic patients from Japan[4]. In another study, Wang *et al*[5] identified a new amino acid change (V385I) that is associated with type 2 diabetic nephropathy. In case of IL-10, polymorphism (-592) in promoter region influence IL-10 and MCP-1 production, which may be an indicator of type 2 diabetic nephropathy risk in Taiwanese patients[6].

Tumour necrosis factor: Gene for tumour necrosis factor (*TNF*)-α is highly polymorphic and is located on chromosome 6p. *TNF*-α -308G/A polymorphism has been implicated in susceptibility to diabetic nephropathy but the results have been contradictory. Studies have shown that polymorphism of the TNF-α gene at the -308 position is significantly related to an increased risk of kidney failure in patients with type 2 diabetes (T2DM)[7,8]. In contrast to this, Lindholm *et al*[9], demonstrated that the allele frequencies of *TNF* -308 G→A and *LTA* T60N polymorphisms were similar in type 1 diabetic patients with and without diabetic nephropathy and no differences were observed between type 2 diabetic patients with and without diabetic nephropathy in allele or haplotype frequencies of the studied polymorphisms. In a recent meta analysis it was demonstrated that A allele of *TNF*-α -308G/A polymorphism might be protective against diabetic nephropathy but with ethnic selectivity[10].

Genetic variants of extracellular matrix components

Collagen, type Ⅳ, alpha 1: The Collagen, type Ⅳ, alpha 1 (*COL4A1*) provides instructions for making one component of type Ⅳ collagen, which is a flexible protein important in the structure of many tissues throughout the body. Two single nucleotide polymorphism's in intron 1 (rs614282 and rs679062) showed significant association with diabetic nephropathy[3]. Other studies on genetic variants of *COL4A1* gene have shown contradictory results where Krolewski *et al*[11] showed that a polymorphic *Hin*dⅢ restriction site was associated with increased risk for progression to diabetic nephropathy and contradictory to it, Chen *et al*[12] found no association in larger sample size.

Laminins: Laminins (LAM) are extracellular matrix glycoproteins which are the major noncollagenous constituent of basement membranes. They are involved in various biological processes like cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis. Ewens *et al*[3] found a gene variant (rs3734287) located in *LAMA4* gene's intronic region and Asn837Asn variant (rs20557) in *LAMC1* gene, to be significantly associated with diabetic nephropathy.

Matrix metalloproteinase 9: Two studies conducted by Maeda *et al*[13] and Hirakawa *et al*[14] had found evidence for association between diabetic nephropathy and Short Tandem-Repeat Polymorphism in the promoter microsatellite locus (D20S838) of Matrix metalloproteinase 9 (*MMP9*) in Japanese and Caucasian type 2 diabetic patients, respectively. In contrast, Ewens *et al*[3], found no evidence of association between any D20S838 allele with

diabetic nephropathy. However, significant association was seen between diabetic nephropathy and rs11697325, an SNP located 8.2 kb 5' of *MMP9*[13,14].

Gene variants of renal function components

Angiotensin Ⅰ -converting enzyme: Angiotensin-converting enzyme is a potent vaso-constrictor and increases blood pressure. Polymorphisms in this gene are clearly associated with circulating angiotensin Ⅰ -converting enzyme (ACE) levels and studies have shown positive association between the *ACE* DD allele and type 1 diabetic nephropathy[15-17]. This study is in confirmation to a meta analysis where subjects with the Ⅱ genotype had a 22% lower risk of diabetic nephropathy than carriers of the D allele suggesting a genetic association of the *ACE* Ⅰ /D polymorphism with diabetic nephropathy in type Ⅰ[18] and type Ⅱ patients[19]. Although a large meta-analysis failed to confirm the diabetic nephropathy association in white individuals[20] but another report from the European Rational Approach for the Genetics of Diabetic Complications (EURAGEDIC) Study Group detected evidence for association of several *ACE* polymorphisms (including the "D" deletion allele) in a large case-control study, with somewhat consistent findings in a family-based transmission disequilibrium testing analysis[15]. A study on Iranian population also showed similar results where neither the DD genotype nor the D allele was associated with diabetic nephropathy[21].

Angiotensinogen and angiotensin Ⅱ receptor type 1 and 2 (AGT and AGTR1, AT2R): A meta-analysis conducted by Mooyaart *et al*[22], found no association between gene variants in the renin-angiotensin system, such as the rs699 variant of angiotensinogen (*AGT*) and the rs5186 polymorphism of angiotensin Ⅱ receptor type 1 (*AGTR1*), with diabetic nephropathy. In contrast, a recent study on angiotensin type 2 receptor (*AT2R*) found an association between the AT2R -1332 G:A polymorphism and the risk of diabetic nephropathy in females[23].

Gene variants of endothelial function and oxidative stress

Nitric oxide synthase 3 (NOS): It is considered as a potential candidate gene for diabetic nephropathy susceptibility[24,25]. Three polymorphisms in this gene *G894T* missense mutation (rs1799983), a 27-bp repeat in intron 4, and the T786C single nucleotide polymorphism (SNP) in the promoter (rs2070744) have been found to be associated with diabetic nephropathy susceptibility[26-30].

The G894T variant was found to increase the risk of macroalbuminuria and progression from microalbuminuria to macroalbuminuria, with declining glomerular filtration rate as serum creatinine value rises progressively, culminating in nephropathy[31,32] However, these results have been contradictory and not all studies support this association[33-35]. Recent studies on different gene variants observed that there was an association between *eNOS*-4b/a polymorphism and the risk of type 2 diabetic nephropathy[36,37] while others suggested that there was no significant association[38]. Recently, a report from Arab population also failed to find an association between *eNOS* gene G894T polymorphism with the risk of type 2 diabetic nephropathy[39].

Catalase: This enzyme protects the cell from oxidative damage by reactive oxygen species (ROS) by breaking down hydrogen peroxide to water and oxygen. Two variants of catalase (*CAT*) gene one located in the 5'-untranslated region (rs1049982) and other located in intron 1 (rs560807) were found to be involved with the risk of type 1 diabetic nephropathy[3].

Superoxide dismutase 2 (MnSOD/SOD2): Manganese superoxide dismutase (MnSOD) protects the cells from oxidative damage by scavenging free radicals. The study on valine/alanine polymorphism in *MnSOD* gene (V16A, rs4880) revealed that, the subjects with Val allele were associated with increased risk of type 1 diabetic nephropathy[40]. The result of this study is in agreement with results by other studies[41,42], who found lower frequency of the Ala allele in Japanese and Korean type 2 diabetic patients with diabetic nephropathy as compared to controls. This Val allele was more common in the Japanese and Korean populations (85%-90%) than the northern Caucasian population (50%) and is strongly associated with diabetic nephropathy. A recent study showed that *SOD2* Val16Ala polymorphism was significantly associated with macroalbuminuria in a sample of Mexican type 2 diabetes patients where the frequency of the TT genotype was 6.7% higher in participants with macroalbuminuria than in the normoalbuminuria group[43].

Gene variants of glucose and lipid metabolism

Adiponectin (ADIPO): It is a adipocytokine encoded by adiponectin gene with substantial anti-inflammatory properties and is a major modulator of insulin resistance and dyslipidemia. The minor allele (A) in intron 1 (rs182052) of adiponectin gene was found to be associated with diabetic nephropathy in an African American population[44]. Another study showed the strongest association between a polymorphism in the promoter region of adiponectin gene, rs17300539 (*ADIPOQ*_prom2/rs17300539 G > A) and diabetic nephropathy where the A-allele was found to increase the risk for nephropathy while the G-allele was found to be protective against the same. This association was found to be significant in Denmark and marginal in France but was not significant in Finland[45]. However, in a study conducted by Mooyaart *et al*[22], found no link between rs17300539 of adiponectin gene with diabetic nephropathy.

Apolipoprotein E: The apolipoprotein gene has been found to be associated with increased susceptibility to diabetic nephropathy[46]. It is a triallelic gene consisting of ε2, ε3, and ε4 alleles which are defined by a single amino acid substitution at two sites[47]. Amongst these alleles, E2

and the E4 allele of apolipoprotein E (*APOE*) gene were found to be associated with diabetic nephropathy in a meta-analysis[22] where, E2 allele lead to an increased risk of diabetic nephropathy and the E4 allele was found to have a protective effect. However, the influence of three-allelic variations in the *APOE* gene for the development of diabetic nephropathy may be weak or moderate, but not strong[48].

Aldose reductase: This enzyme catalyzes the reduction of glucose to sorbitol in the first step in polyol pathway of glucose metabolism. Ko *et al*[49] first identified seven alleles at the locus of the (AC)n dinucleotide repeat sequence upstream of Aldose reductase gene (*AKR1B1*). Several studies have demonstrated a correlation between the Z-2 allele (23 AC repeats) and susceptibility to an increased risk of diabetic nephropathy in both type1 and type 2 diabetes mellitus[50,51]. Heesom *et al*[52] also showed that individuals with the Z+2 allele are more than seven times less likely to develop diabetic nephropathy than those without this gene variant. A meta-analysis found a correlation between the (AC)n dinucleotide repeat polymorphism and the occurrence of diabetic nephropathy in Caucasian type 1 diabetic subjects in contrast to type 2 diabetic subject population in which neither the risk ZK2 allele nor the protective ZC2 allele in type 1 diabetic subjects appeared to have an effect on nephropathy in type 2 diabetic subjects[53]. A second polymorphism in this gene has been observed at position-106 of its promoter region. This polymorphism in aldose reductase gene was also found to be associated with nephropathy in type 1 and type 2 diabetic patients[54]. This polymorphism was also found to be involved in the early development of microalbuminuria in Finnish T2DM patients and was proposed as a risk factor for development of nephropathy in T2DM patients with poor glycaemic control[55].

Glucose transporter 1: Glucose transporter 1 (*GLUT1 or SLC2A1*) is the major facilitative glucose transporter in glomerular mesangial cells. Experimental evidence suggests that *GLUT1* may be associated with hypertensive glomerulopathy[56]. Ng *et al*[57], showed that SNPs at the *GLUT1* (XbaI -intron 2 and HaeIII SNPs-exon 2) were associated with susceptibility to diabetic nephropathy in type 1 diabetes. A meta-analysis on the other hand demonstrated a significant association between the another polymorphic site *SLC2A1* XbaI in *GLUT1* gene with Diabetic nephropathy[58].

A study of those with type 1 diabetes examined six *GLUT1* SNPs and found homozygosity for the XBAI A allele and for minor allele(C-to-T) of the enhancer-2 SNP1 (ENH2 SNP) was associated with diabetic nephropathy in type 1 diabetes[57] whereas, no statistically significant association was found between *Xba* I gene variants and type 2 diabetic nephropathy[57]. Among the gene variants identified in the *GLUT1* putative enhancer elements, the AA genotype of enhancer-2 SNP1 (rs841847) is a "risk genotype"[57] and that the TT genotype of the 5' promoter region (rs710218) was associated with nephropathy[59]. Moreover, the patients with the AG haplotype (rs841847-rs841853) have an increased risk of diabetic nephropathy and the TT haplotype (rs710218-rs841853) was more frequent in nephropathic patients. These findings showed that two haplotypes (composed of rs1385129-rs841847-rs841848) are associated with a 4.4 and 2.6-fold increased risk of nephropathy in the Tunisian T2DM patients[60].

However, the results of various case-control studies on *GLUT1* gene variants and their association with diabetic nephropathy have been inconsistent showing heterogeneity between studies[57,61-63].

Peroxisome proliferator-activated receptor gamma 2: Peroxisome proliferator-activated receptor gamma 2 (PPARG2) is a receptor expressed selectively in the adipose tissue where it modulates the expression of genes involved in adipocyte differentiation and glucose homeostasis. The *Pro12Ala* gene variant was associated with lower albumin excretion rates among Ala12 carriers with type 2 diabetic nephropathy. Thus it could be suggested that Pro12Ala polymorphism may be protective against the disease since microalbuminuria is considered to be a risk factor for diabetic nephropathy[64]. This study was confirmed by Pollex *et al*[65] who showed that the Ala12 allele carriers have 1.5-fold reduction of the albumin/creatinine ratio and thus reduced occurrence of microalbuminuria. A recent meta-analysis showed that Pro12Ala polymorphism in *PPARγ2* gene is not a risk factor for diabetic nephropathy in type 2 diabetes[66].

Other gene variants involved

Apart from the above mentioned genes and their variants, there are various other gene variants for various genes like genes coding for growth factor, inflammatory factors, transcription factors, cytoskeletal proteins, components of immune system etc which have also been implicated in predisposing an individual to the risk of developing diabetic nephropathy. Some of these gene variants are discussed in Table 1.

CONCLUSION

Diabetic nephropathy is progressively becoming a major challenge for the health care system, since it is as yet poorly understood in many aspects. It is the leading cause of premature death in young diabetic patients (between 50 and 70 years old). It is a heterogenous and a multifactorial disease with several genes, proteins and environmental factors contributing to its risk. Due to the growing burden of the disease in diabetic patients, it is important to identify diabetic nephropathy predictors, for the proper management of this disease. Genetic susceptibility has been proposed as an important factor for diabetic nephropathy. Multiple genes are involved in pathogenesis of diabetic nephropathy, with several allelic polymorphisms having demonstrable effects in the devel-

Table 1 Gene variants associated with diabetic nephropathy

Gene category	Gene name	Gene variant symbol	Location	Phenotype	Ref.
Growth factors	Insulin-like growth factor 1	IGF-1	12q23.2	Type 1 DN	[3]
	IGF-binding protein 1	IGFBP1	7p14	Type 2 DN	[67]
	Transforming growth factor-β receptor Ⅱ	TGF β R2	3p24.1	Type 1 DN	[3]
	TGF-β receptor Ⅲ	TGF β R3	1p22.1	Type 1 DN	[3]
Matrix metalloproteinases and dipeptidases	Tissue inhibitor of metalloproteinase 3	TIMP3	22q12.3	Type 1 DN	[3]
	Matrix metalloproteinase 9	MMP9	20q13.12	Type 1 DN	[3]
	Carnosinase	CNDP1	18q22.3	Type 2 DN	[68,69]
Transcription factors	Transcription factor 2, hepatic	HNF1B1/TCF2	17q12	Type 1 DN	[3]
	Neuropilin 1	NRPI	10p11.22	Type 1 DN	[3]
	Protein kinase C β 1	PRKCBI	16p12.1	Type 1 DN	[3]
	Upstream transcription factor 1	USFI	1q23.3	Type 1 DN	[3]
Other genes	Engulfment and cell motility factor	ELMO1	7p14	Type 2 DN	[70-72]
	Cytochrome b, α polypeptide	p22phox	16q24.3	Type 1 DN	[3]
	Glutathione peroxidase 1	GPXI	3p21.3	Type 1 DN	[3]
	B-cell leukemia/lymphoma 2 (bcl-2)	BCL2	18q21.33	Type 1 DN	[3]
	Aquaporin 1	AQP1	7p14.3	Type 1 DN	[3]

opment and progression of the disease thus contributing to the overall risk. These polymorphisms in several genes distributed widely across the human genome, each with a modest effect size, may be causal or protective factors in the development and progression of diabetic nephropathy. The combining of the various gene polymorphism studies in diabetic nephropathy related genes with recent researches/developments in the fields of human genomics, proteomics and bioinformatics would help in early diagnosis, treatment and prevention by giving us a better understanding of the pathogenesis of diabetic nephropathy. Identification of genes associated with diabetic nephropathy could provide a powerful tool for identifying patients at risk of developing diabetic nephropathy in the late future. In this context research efforts have been invested worldwide to identify the susceptibility gene for diabetic nephropathy. Epidemiologic studies and candidate-gene-based association studies are the most common approaches employed to identify susceptibility genes for diabetic nephropathy. Many genes were found to be associated with the disease but the results had been inconsistent and most of the candidate genes for diabetic nephropathy remain still to be identified. The inclusion of genetic studies in design and analysis of drug trials could lead to development of genetic biomarkers that predict treatment response. Thus, collaborative efforts are needed to achieve substantial findings in the study of genetics of diabetic nephropathy which could give us a better prospective of biochemical and molecular mechanism of disease on the whole. Early identification of at risk patients will facilitate earlier intervention; ultimately delaying and reducing the impact of nephropathy remain still to be identified. Thus, collaborative efforts are needed to achieve substantial.

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REVIEW

Incretin-based therapies in prediabetes: Current evidence and future perspectives

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Abstract

The prevalence of type 2 diabetes (T2D) is evolving globally at an alarming rate. Prediabetes is an intermediate state of glucose metabolism that exists between normal glucose tolerance (NGT) and the clinical entity of T2D. Relentless β-cell decline and failure is responsible for the progression from NGT to prediabetes and eventually T2D. The huge burden resulting from the complications of T2D created the need of therapeutic strategies in an effort to prevent or delay its development. The beneficial effects of incretin-based therapies, dipeptidyl peptidase-4 inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists, on β-cell function in patients with T2D, together with their strictly glucose-depended mechanism of action, suggested their possible use in individuals with prediabetes when greater β-cell mass and function are preserved and the possibility of β-cell salvage is higher. The present paper summarizes the main molecular intracellular mechanisms through which GLP-1 exerts its activity on β-cells. It also explores the current evidence of incretin based therapies when administered in a prediabetic state, both in animal models and in humans. Finally it discusses the safety of incretin-based therapies as well as their possible role in order to delay or prevent T2D.

Key words: Type 2 diabetes; Prediabetes; Impaired fasting glucose; Impaired glucose tolerance; Glucagon-like peptide-1; Dipeptidyl peptidase-4 inhibitors; Glucagon-like peptide-1 receptor agonists

Core tip: The beneficial effects of incretin-based therapies on β-cell function in patients with type 2 diabetes (T2D) suggested their possible use in individuals with prediabetes, when greater β-cell mass and function are preserved. Both dipeptidyl peptidase-4 inhibitors and glucagon-like peptide-1 receptor agonists have demonstrated improvements on β-cell function both in preclinical studies and short-term clinical studies. Until future date for their safety are available, large, long term, prevention trials will be required in order to determine whether they can stabilize or reverse β-cell loss and promote a sustained reduction in the development of T2D in this population.

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INTRODUCTION

The prevalence of type 2 diabetes (T2D) is evolving globally at an alarming rate[1]. It is estimated that by the year 2030 approximately 366 million people will have diabetes and more than 90% of them T2D[1,2]. Prediabetes is an intermediate state of glucose metabolism that exists between normal glucose tolerance (NGT) and the clinical entity of T2D[3]. It encompasses both impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). IFG is defined by a fasting plasma glucose of 100 mg/dL to 125 mg/dL, while IGT is defined by a 2 h plasma glucose concentration of 140 mg/dL to 199 mg/dL after a 75 g

oral glucose tolerance test (OGTT)[3,4]. Furthermore, the American Diabetes Association suggested that glycated hemoglobulin (A1C) between 5.7% and 6.4% can also be used for the diagnosis of prediabetes, considering that A1C test must be performed by a method that is certified by the National Glycohemoglobin Standardization Program and standardized or traceable to the Diabetes Control and Complications Trial reference assay[4]. Approximately 471 million people worldwide (8% of the world's adult population) are estimated to have IGT by the year 2035[1].

Individuals with IGT have moderate to severe muscle insulin resistance and normal to slightly decreased hepatic insulin sensitivity. They are characterized by defects in both early (0-30 min) and late-phase (60-120 min) of insulin secretion to an oral glucose load[5]. Individuals with IFG have moderate hepatic insulin resistance with normal muscle insulin sensitivity and decreased basal and early phase of insulin secretion[5]. The Veterans Administration Genetic Epidemiology Study and the San Antonio Metabolism (SAM) study have shown a progressive decline in pancreatic β-cell function in individuals with prediabetes[6,7]. The SAM study has demonstrated that when the 2 h plasma glucose during an OGTT was 180-190 mg/dL, β-cell function had already declined by 75% to 80%[6]. Eventually, approximately 20%-34% of the individuals with IFG or IGT progress to T2D over five to six years, while those with combined IFG and IGT have a cumulative incidence of 38%-65%, especially if they have low insulin secretion and severe insulin resistance[8,9]. Relentless β-cell decline and failure is responsible for the progression from NGT to IGT and eventually T2D.

A two to three fold greater increase in plasma insulin response is observed after glucose ingestion compared to a parenteral isoglycemic glucose infusion. This phenomenon was defined as the incretin effect; it accounts for approximately 70%-80% of total insulin release after oral glucose administration[10,11]. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are the two major incretins described; they account for approximately 90% of the incretin activity[12]. GLP-1 contributes in the overall maintenance of glucose homeostasis through the reduction of glucagon secretion, slowing of gastric emptying and control of body weight, by its appetite suppressant effect[10,11]. GLP-1 levels are significantly decreased in T2D (approximately 50% compared to healthy individuals)[10,13,14]. GIP levels are found to be elevated in patients with T2D as a result of resistance to its biological effects. Sensitivity of β-cells can be resorted after normoglycemia is established, suggesting that resistance to GIP is a manifestation of glucotoxicity[15].

Impairment in incretin hormone secretion/activity in individuals with prediabetes has been reported, although data are not consistent[16-22]. However, reduced GLP-1 levels were reported in the majority of these studies and mainly in subjects with isolated IGT or combined IFG and IGT; early phase GLP-1 response was found to be severely diminished[17-22]. Interestingly, Toft-Nielsen *et al*[22] have shown that during the progression from NGT to IGT and eventually T2D, there is a progressive decline in GLP-1 levels. Early GLP-1 therapy was suggested to preserve β-cell function in subjects with IGT or mild T2D[23].

Native GLP-1 is rapidly inactivated (halfe-life of 1-2 min) by the ubiquitously expressed proteolytic enzyme dipeptidyl peptidase-4 (DPP-4)[10]. The DPP-4 inhibitors are a class of oral antidiabetic agents that improve glycemic control, in patients with T2D, by increasing both GLP-1 and GIP concentrations[24]. GLP-1 receptor (GLP-1R) agonists mimic the actions of GLP-1 and are resistant to DPP-4 degradation; they have achieved significantly lower A1C values in patients with T2D that were associated with significant weight reduction[25]. Studies in cell cultures and animal models demonstrated that both DPP-4 inhibitors and GLP-1R agonists have trophic effects on pancreatic β-cells. Specifically they enhance β-cell proliferation, regeneration and differentiation; thus they increase β-cell mass. They also inhibit β-cell apoptosis, including human β-cells, through inhibition of the caspase pathway[24-26]. The identification of their antiapoptotic properties, combined with observations of β-cell function preservation and sustained glycemic control during their administration, suggested their possible use as early in the clinical course of T2D as possible or even earlier in order to prevent the onset of this disease[27]. The present paper summarizes the main molecular intracellular mechanisms through which GLP-1 exerts its activity on β-cells. It also explores the current evidence of incretin-based therapies, DPP-4 inhibitors and GLP-1R agonists, when administered in a prediabetic state both in animal models and in humans. Finally it discusses the safety of incretin-based therapies, as well as their possible role in order to delay or prevent T2D.

MAIN MOLECULAR INTRACELLULAR MECHANISMS OF GLP-1 ACTIVITY ON THE PANCREATIC β-CELL

Increased glucose levels are first transported into the β-cell by the type 2 facillitative glucose transporter (GLUT-2) and are phosphorylated by glucokinase to glucose-6-phosphate, promoting an increased rate of aerobic glycolysis; this in turn generates substrates (mainly pyruvate) for mitochondrial oxidative metabolism. Glycolytic and mitochondrial respiration promotes an increased cytosolic adenosine triphosphate (ATP)/adenosine diphosphate (ADP) concentration[28]. This major cellular metabolic signal provides the link between glucose stimulus and insulin secretion. The increase of ATP/ADP ratio promotes the closure of ATP-sensitive K^+ channels (K_{ATP}), thereby initiating plasma membrane depolarization, activation of voltage-dependent Ca^{2+} channels (VDCCs), Ca^{2+} influx and an increase in the intracellular Ca^{2+} concentration. This in turn stimulates the granules that contain insulin and promotes their release into the

blood compartment. Repolarization of β-cells is mainly mediated by Ca^{2+}-sensitive voltage-depended K^+ (K_{Ca}) channels and voltage-dependent K^+ (Kv) channels. These channels open after glucose-induced membrane depolarization so as to restore the outward flux of K^+[29].

GLP-1 is a 30-amino acid peptide produced in the intestinal epithelial L-cells of the distal ileum and colon by differential processing of the proglucagon gene from the prohormone convertase PC1/3[30]. GLP-1 binds to GLP-1R, a class 2 G protein-coupled receptor, in the cell membrane of the pancreatic islets[31]. Through this receptor it mainly exerts its insulinotropic activity, which is strictly glucose-depended. Specifically, it stimulates adenylate cyclase resulting in the production of cyclic adenosine 3',5'-monophosphate (cAMP). Downstream effectors of cAMP include protein kinase A and the cAMP-regulated guanine nucleotide exchange factor Ⅱ. Through the activation of these two important cellular pathways GLP-1 enhances and amplifies insulin secretion *via* its effects on ATP/ADP concentration ratio, K_{ATP} channels, Kv and K_{Ca} channels, VDCCs, Ca^{2+} influx and intracellular concentrations and insulin granule exocytosis or priming[32,33]. In this way GLP-1 restores glucose-depended insulin secretion in metabolically compromised β-cells; it promotes the induction of glucose competence (Figure 1)[34,35].

In addition to its insulinotropic effects, GLP-1 acts as β-cell growth factor. After binding to its receptor, GLP-1 induces the transactivation of the epidermal growth factor receptor, which activates phosphatidylinositol-3 kinase (PI3-K) and its downstream targets protein kinase B (PKB/Akt), extracellular signal-related kinase, p38 mitogen-activated protein kinase (MAPK) and protein kinase Cζ [36,37]. Through these pathways GLP-1 exerts its action on β-cell proliferation and survival. Moreover GLP-1 promotes an increased expression and activity of the pancreatic and duodenal homeobox-1 (*PDX-1*) gene; hence it increases total PDX-1 levels and promotes its translocation to the nucleus[38]. PDX-1 is of major significance for most of the proliferative, glucoregulatory and cytoprotective actions of GLP-1. It regulates the expression of genes important for β-cell function such as insulin, GLUT-2 and glucokinase. It also replenish β-cell insulin stores and in a long term basis it prevents β-cell exhaustion[38-42]. Moreover, GLP-1 stimulates β-cell proliferation through CREB-mediated *Irs2* gene expression, leading to activation of PI3-K/PKB signaling pathway[43]. Its proliferative activity was also related to insulin growth factor (IGF)-1 expression and autocrine IGF-2 secretion by the β-cell[44]. Furthermore, GLP-1 prevents β-cell apoptosis, induced by a variety of cytotoxic stimuli, and enhances β-cell survival[26,45,46].

DPP-4 INHIBITORS IN A PREDIABETIC STATE

Vildagliptin

Studies organized in animal models: Vildagliptin (LAF237) is an oral agent that inhibits DPP-4 and increases both active GLP-1 and GIP levels; it achieved improved glycemic control in patients with T2D[47]. Five-week-old female C57BL/6J mice were fed with a high-fat diet, as a model of IGT and T2D, or a normal diet for 8 wk[48]. After 4 wk, the mice were treated with vildagliptin in their drinking water (approximately 3 μmol per day per mouse). Controls were given only water. All mice were subjected to an OGTT after 4 wk of treatment. In both high-fat diet-fed mice and the normal diet-fed mice, administration of vildagliptin improved glucose tolerance in association with markedly augmented insulin secretion.

Vildagliptin was also administered in anesthetized obese insulin resistant cynomolgus monkeys in a dose of 1 μmol/kg[49]. Each animal received two OGTTs 45 min after oral administration of vildagliptin or vehicle, 3 wk apart. Plasma DPP-4 activity was inhibited by 82% with vildagliptin therapy ($P < 0.001$) and remained suppressed throughout the duration of the OGTT. Peak plasma GLP-1 levels in the vildagliptin group were significantly higher than those in the vehicle-treated animals, after the glucose load was given ($P < 0.001$). Vildagliptin reduced glucose excursions during OGTTs compared to the vehicle ($P < 0.05$). There was also a trend towards an enhanced insulinogenic response to glucose after vildagliptin therapy.

Clinical studies: Although incretins are stimulated during an oral challenge, it was postulated that due to the long half-life of DPP-4 inhibitors, basal levels of active GIP and GLP-1 could play a role in the improvement of β-cell function in individuals with IFG. Vildagliptin was investigated in a single-blind, single-treatment design study, in which 22 individuals with IFG were enrolled. The drug was administered in a dose of 100 mg daily for 6 wk. Two weeks of placebo treatment before (running period) and after (washout period) the 6 wk were also studied[50]. Treatment with vildagliptin resulted in a slight increase in fasting GIP but not GLP-1 levels, while marked increases of both intact GLP-1 and GIP levels during a meal tolerance test were reported. Fasting plasma glucose (FPG) levels were not significantly reduced. Incremental area under the curve (AUC) of glucose and 2 h glucose decreased after a meal tolerance test. Although AUC of C-peptide and insulin responses did not change significantly, when the decrease in glucose levels was taken into consideration, both markers were improved. Since a formal OGTT was not performed in the population enrolled, the possibility that some individuals had combined IFG and IGT could not be excluded. The disposition index (DI) was increased by 69% and insulin sensitivity by 25% after an intravenous glucose tolerance test (IVGTT), suggesting an improvement of β-cell function when no dynamic change in incretin release would be expected to occur. However, after the 2-wk washout period, all the beneficial effects observed returned to baseline levels.

In a multicenter 12-wk double-blind study 179 individuals with IGT were randomized to receive either

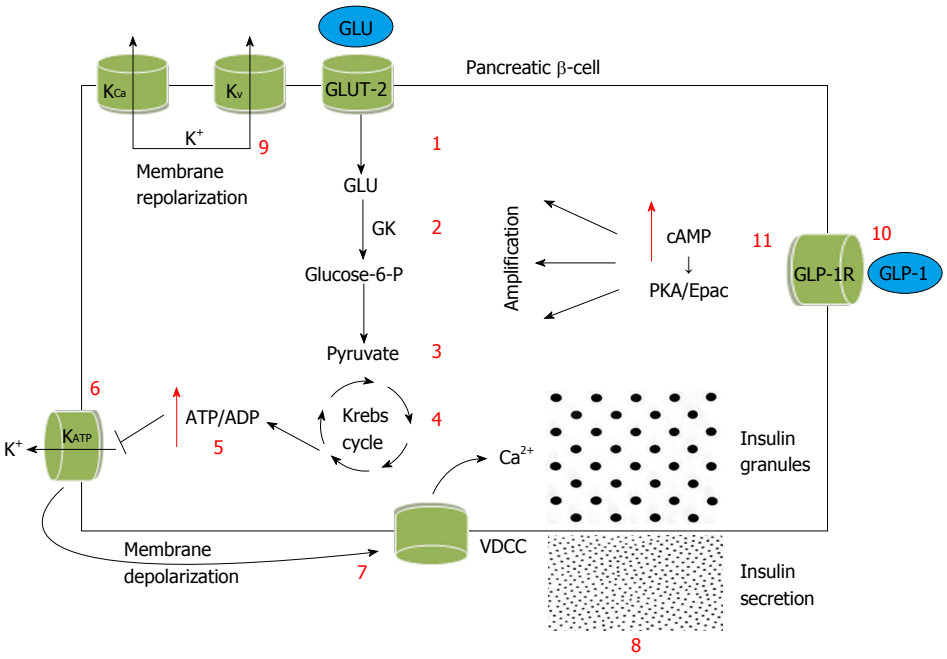

Figure 1 Glucagon-like peptide-1 and the β-cell: Amplification of the glucose-stimulated insulin secretion. Increased glucose levels are transported into the β-cell by GLUT-2. They are phosphorylated by GK to glucose-6-P, promoting an increased rate of aerobic glycolysis. Pyruvate is the main substrate for mitochondrial oxidative metabolism. Increased cytosolic ATP/ADP concentration is the major cellular metabolic signal between the glucose stimulus and insulin secretion. It promotes the closure of K_{ATP} channels, thereby initiating plasma membrane depolarization, activation of VDCCs, Ca^{2+} influx and an increase in the intracellular Ca^{2+} concentration. This in turn stimulates the granules that contain insulin and promotes their release into the blood compartment. Repolarization of β-cells is mainly mediated by K_{Ca} and K_v channels. GLP-1 binds to GLP-1R, a class 2 G protein-coupled receptor, in the cell membrane of the pancreatic cells. Through this receptor it mainly exerts its insulinotropic activity. It promotes increased levels of cAMP through stimulation of adenylate cyclase. Downstream effectors of cAMP are PKA and Epac. Through the activation of these two important cellular pathways GLP-1 amplifies insulin secretion *via* its effects on ATP/ADP concentration ratio, K_{ATP} channels, K_v and K_{Ca} channels, VDCCs, Ca^{2+} influx and insulin granule exocytosis. GLU: Glucose; GLUT-2: Type 2 facillitative glucose transporter; GK: Glucokinase; Glucose-6-P: Glucose-6-phosphate; K_{ATP}: ATP-sensitive K^+ channels; VDCCs: Voltage-dependent Ca^{2+} channels; K_{Ca}: Ca^{2+}-sensitive voltage-depended K^+ channels; K_v: Voltage-dependent K^+ channels; GLP-1: Glucagon-like peptide-1; GLP-1R: Glucagon-like peptide-1 receptor; cAMP: Cyclic adenosine 3',5'-monophosphate; PKA: Protein kinase A; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate.

vildagliptin 50 mg/daily (n = 90) or placebo (n = 89)[51]. Approximately 80% of the patients were IFG and IGT. In individuals receiving vildagliptin there was a marked and sustained increase in active GLP-1 and GIP levels compared to the placebo group (5-fold and almost 2-fold increases in the incremental AUCs for GLP-1 and GIP, respectively). These effects were associated with significant improvements in β-cell function, as estimated by insulin secretion relative to that of glucose (insulin secretory rate AUC0-2 h/glucose AUC0-2 h, mean change between groups 6.1 ± 2.0 pmol/min per meter per millimoles per liter, P = 0.002). Improvements were also reported in α-cell function [glucagon ΔAUC0-2 h, mean change between groups (-3.0 ± 2.0 pmol/L per hour, P = 0.003)]. These beneficial effects contributed approximately to 30% reduction of ΔAUC for glucose. Vildagliptin was well tolerated with a good safety profile and no hypoglycemia was documented.

A three month, double-blind, placebo-controlled study was organized in a population of 48 stable renal transplant recipients, at least six months after transplantation, with newly diagnosed IGT[52]. Participants were randomized to receive 50 mg of vildagliptin, 30 mg of pioglitazone or placebo in a 1:1:1 ratio (16 individuals in each group). There was not any significant difference in corticosteroid therapy between the three groups. Baseline A1C was lowest in the vildagliptin group and higher in the pioglitazone group (P = 0.01). A1C reduction was statistically significant between treatment groups and placebo (placebo *vs* pioglitazone: -0.17% ± 0.33% *vs* +0.09% ± 0.26%; P = 0.013; placebo *vs* vildagliptin: -0.11% ± 0.25% *vs* +0.09% ± 0.26%; P = 0.049). Vildagliptin and pioglitazone reduced the 2 h plasma glucose at three months compared with baseline (vildagliptin: -20 ± 24 mg/dL; P = 0.002 and pioglitazone: -23 ± 29 mg/dL; P = 0.004), while only pioglitazone slightly reduced FPG.

Sitagliptin

Studies organized in animal models: Sitagliptin is the first DPP-4 inhibitor introduced in clinical practice[53]. Sitagliptin and glyburide were administered in obese prediabetic spontaneously hypertensive rat-obese (SHROB) in order to investigate whether it could reverse the metabolic abnormalities in the secretion of both insulin and glucagon[54]. Sitagliptin was found to normalize glucose tolerance following an OGTT, at least as effective as glyburide, in this rat model of metabolic syndrome and prediabetes. Sitagliptin also restored the first phase of insulin secretion after an OGTT more effectively than glyburide. Fasting glucagon levels, which were elevated

in the SHROB model, were normalized after 5 wk of sitagliptin therapy. Fasting insulin and liver glucogen levels were not affected by both drugs. It was suggested that if sitagliptin actions could extend to human prediabetics, then sitagliptin might delay the onset of diabetes[54].

Sitagliptin was also administered in a mouse model of diet-induced obesity with increased FPG and postprandial hyperinsulinemia[55]. It was reported that 12-wk of sitagliptin therapy improved glucose tolerance, reduced FPG, and lowered plasma insulin in randomly fed mice compared with untreated insulin-resistant obese mice. A significant reduction in glucose excursions during an intraperitoneal glucose tolerance test was found. Sitagliptin was also shown to induce a change in the islet size distribution. Specifically, a significantly higher percentage of small islets and a reduced relative percentage of very large islets (due to the very high-fat diet) was demonstrated. This result may explain the better insulin secretory response observed after sitagliptin therapy in response to an *in vitro* glucose challenge.

An animal model with clinical and metabolic characteristics similar to those of individuals with IGT was recently studied[56]. Fructose administration to normal rats for 21 d induced insulin resistance, IGT, hypertriglyceridemia and decreased β-cell mass, due to an increased percentage of apoptosis. The control group was consistent of rats that were fed with a standard commercial diet. Homeostasis model assessment for insulin resistance (HOMA-IR) and for β-cell function (HOMA-β) decreased to almost control values after sitagliptin therapy. Sitagliptin significantly increased β-cell mass by 68%, attaining values close to those measured in standard commercial diet fed rats; inhibition of β-cell apoptosis was the main cellular mechanism for this effect. These changes were associated with normalization of IGT and liver triacylglycerol content.

Clinical studies: In a double blind placebo-controlled trial 22 individuals with IFG, after a baseline meal study, received sitagliptin 100 mg daily (n = 11) or placebo (n = 11) over an 8-wk treatment period[57]. They underwent a second meal study at the end of the treatment period. Sitagliptin did not alter fasting but increased postprandial intact GLP-1 concentrations, while total postprandial GLP-1 concentrations were reduced. Both fasting and postprandial glucose values were unchanged with sitagliptin therapy. Although sitagliptin resulted in a slight improvement in β-cell function (a slightly increased DI was found), this was not sufficient to alter glucose uptake and production and overcome the defect on insulin action. It was speculated that the limited ability of DPP-4 inhibitors to increase insulin secretion in IFG could be due to their glucose depended mechanism, since glucose concentrations are only modestly elevated in IFG. This speculation can also explain the differing effectiveness of sitagliptin on postprandial concentrations in this study compared to other studies in individuals with IGT, with higher postprandial glucose concentrations.

A four week open-label, parallel group study investigated the effects of sitagliptin on insulin secretion and endogenous glucose production in individuals with IFG and no history of prior antidiabetic therapy[58]. Twenty-three individuals with either IFG (n = 10) or NGT (n = 13) were studied by a fasting glucose test and OGTT. All participants received open-label sitagliptin 100mg once daily for 4 wk. Treatment with sitagliptin resulted in a small but significant decrease in FPG compared to baseline in both groups ($P < 0.05$). Endogenous glucose production was unchanged after 4 wk of sitagliptin therapy. Administration of sitagliptin did not altered insulin or glucose excursions in the post-intervention OGTT, but did increase AUC for active GLP-1 and C-peptide compared to baseline levels ($P < 0.01$ for both). Insulin sensitivity and β-cell response indices remained unchanged after administration of sitagliptin.

Beta-cell function in Glucose abnormalities and Acute Myocardial Infarction was a 12-wk multicentre, double-blind, randomized, parallel group study that investigated the effects of sitagliptin 100 mg daily (n = 34) compared to placebo (n = 37) in 71 patients with acute coronary syndrome having IGT or T2D[59]. Investigation of β-cell function was achieved using the insulinogenic index (IGI) derived from an OGTT and acute insulin response to glucose (AIRg) after a frequently sampled IVGTT. At the time of randomization 71% and 62% of the individuals in the sitagliptin and the placebo group had IGT, while 29% and 38% had T2D, respectively. IGI increased significantly, from baseline to 12 wk (9.9 pmol/mmol to 85.0 pmol/mmol) in the sitagliptin group compared to the placebo group (66.4 pmol $mmol^{-1}$ to 58.1 pmol/mmol, P = 0.013). The AIRg increased significantly in the sitagliptin group compared to the placebo group: 1909 pmol L^{-1} per minute *vs* 1043 pmol/L per minute ($P < 0.0001$). During the OGTT and the frequently sampled IVGTT, glucose levels were significantly lower in the sitagliptin arm compared to the placebo arm. Immediate insulin response was higher after sitagliptin therapy, while it remained unchanged after placebo. By 12 wk, 76%, 18% and 6% of the participants in the sitagliptin group had NGT, IGT and T2D respectively. In the placebo arm 41%, 35% and 24% of the participants had NGT, IGT and T2D respectively.

Other DPP-4 inhibitors

Alogliptin is the newest DPP-4 inhibitor approved for T2D therapy, either alone or in combination with other antidiabetic agents[60]. It was administered alone or in combination with voglibose in prediabetic *db/db* mice[61]. Specifically, 6 wk old prediabetic db/db mice were fed with a powder CE-2 diet containing 0.001% voglibose alone (equivalent to 1.8 mg/kg per day), 0.03% aloglitpin alone (equivalent to 72.8 mg/kg per day), or combination of both agents (equivalent to alogliptin: 53.8 mg/kg per day + voglibose: 1.8 mg/kg per day) for 27 d. Control *db/db* and non-diabetic *db/+* mice were fed by a drug-free powder CE-2 diet (vehicle). Plasma DPP-4 activity

was reduced significantly by 18%, 72% and 80% and plasma active GLP-1 levels were increased significantly by 1.8, 4.5 and 9.1-fold in voglibose, alogliptin and combination treated *db/db* mice, compared with vehicle treated *db/db* mice, respectively. Pancreatic insulin content was increased significantly by 3.4, 1.8 and 8.5-fold and A1C was reduced significantly by 1.6%, 0.5% and 2.1% in voglibose, alogliptin and combination treated *db/db* mice, compared with vehicle treated *db/db* mice, respectively. Although quantitative analysis was not preformed, combination treatment resulted in an increased pancreatic insulin staining, PDX-1 staining and GLUT2 membrane localization in β-cells. It also maintained normal distribution of β/α-cells in islets; it was suggested that this combination could preserve pancreatic β-cells in *db/db* mice[61]. The combination of alogliptin and pioglitazone was also found to improve glycemic control and increase pancreatic insulin content in *ob/ob* mice; however the addition of alogliptin to pioglitazone therapy did not contributed to the prevention or the delay of T2D onset in UCD-T2DM rats[62,63].

The effects of chronic administration of the DPP-4 inhibitor FE 999011 were investigated in both obese and insulin resistant fatty Zucker rats and Zucker diabetic fatty (ZDF) rats[64]. Fatty Zucker rats experience mild glucose intolerance, while ZDF become overtly diabetic after 8 wk of age, if they are fed with a diet containing 6.5% of fat. When administered in the fatty Zucker rats, FE 999011 produced a dose-depended reduction in plasma glucose excursion during the OGTT. During an intraduodenal glucose tolerance test it increased GLP-1 levels, while glucose excursions were indistinguishable from that of lean controls. Chronic treatment with FE 999011 in the fatty Zucker rats significantly improved glucose tolerance, as suggested by the decrease in the insulin-to-glucose ratio. Chronic treatment with FE 999011 twice daily in ZDF rats maintained euglycemia for at least 21 d and delayed the onset of diabetes. Lower basal insulin secretion due to improved insulin sensitivity was reported. It also increased basal GLP-1 levels, stabilized food and water intake to prediabetic levels, reduced hypertriglyceridemia and prevented the rise of circulating non-esterified fatty acids (NEFAs). Up-regulation of pancreatic GLP-1 receptor gene expression was also induced by FE 999011.

The DDP-4 inhibitor isoleucine thiazolidine (P32/98) was orally administered for 3 wk to fatty Zucker rats with incipient IGT (iIGT) and 6 wk in rats with manifest IGT (mIGT) in a dose of 21.61 mg/kg (n = 10 per group)[65]. Control rats received the same amount of placebo. Blood glucose day-night profile was significantly reduced in iIGT Zucker rats achieving values near normalization; it was also improved in mIGT rats. P32/98 tended to reduce food intake and body weight gain, as well as non-fasting plasma insulin levels, only in Zucker rats with iIGT. P32/98 bolus before OGTT increased insulin secretion and reduced glucose load both in iIGT and mIGT Zucker rats, suggesting a broad therapeutic efficacy in animal models of IGT. Treatment of isolated pancreatic islets of mIGT Zucker rats with this agent decreased pancreatic insulin content and increased glucose responsiveness, while the β-cell volume density was not improved.

The DPP-4 inhibitor PFK 275-055, a vildaglitpin analogue, was investigated in obese, insulin resistant prediabetic rats for 4 wk in a dose of 10 mg/kg per day[66]. GLP-1 levels increased after PFK 275-055 therapy. Insulin levels were decreased after therapy with this agent, while glucose levels were not affected; an increased β-cell/α-cell ratio was observed. The DPP-4 inhibitor DA-1229 improved pancreatic insulin content, β-cell function and delayed the onset of diabetes in young *db/db* mice[67]. Currently, several studies have been launched and are recruiting individuals in order to explore the possible role of alogliptin and saxagliptin in a prediabetic state[68].

GLP-1R AGONISTS IN A PREDIABETIC STATE

Exenatide

Studies organized in animal models: Exenatide is the synthetic form of the naturally occurring exendin-4, a 39-amino-acid peptide hormone secreted by the salivary glands of the venomous lizard Heloderma suspectum, otherwise known as the Gila monster[69]. It shares 53% structural homology with human GLP-1 and resists inactivation by the DPP-4. In an animal model of profound insulin resistance, IGT, hypertriglyceridemia and decreased β-cell mass, exendine-4 significantly increased β-cell mass by 201%[56]. This effect was achieved after a significant decrease in β-cell apoptosis, although the molecular effect for this activity was not studied. HOMA-IR and HOMA-β indexes remained within normal rage. Normalization of IGT and liver triacylglycerol content was also achieved.

In another well-organized study, exendin-4 was administered to obese prediabetic *db/db* mice at 6 wk of age for 16 d[70]. By the age of 8 wk, vehicle treated mice developed T2D, while mice treated with exendin-4 maintained FPG in the normal range, indicating that this agent delayed the onset of T2D. Improvement in glucose tolerance was also observed with exendin-4. No significant differences were observed between the two groups as far as insulin sensitivity is concerned. Glucose alone induced a two to five-fold increase in insulin secretion in the exendin-4 group, while the pancreas of vehicle-treated mice was unresponsive to the same dose of glucose. A 1.4-fold increase in β-cell mass was observed in exendin-4 mice, which was the result of both increased β-cell proliferation and decreased β-cell apoptosis; these changes were related to higher expression of the protein kinases Akt1 and MAPK.

The ability of exendin-4 to promote β-cell proliferation in young Goto-Kakizaki (GK) rats during the prediabetic state, and therefore prevent the development of T2D when animals become adults, was also explored[71]. Four groups of rats were investigated: two control

groups (control GK and control non-diabetic Wistar rats) and two experimental groups. In the two experimental groups, GK rats received either a subcutaneous daily injection of GLP-1 (400 μg/kg of body weight) or exendin-4 (3 μg/kg of body weight) for five days (day's two to six) after their birth. Animals were killed seven days or two months after birth. Seven days after their birth GK rats showed significantly higher pancreatic insulin content and doubling of β-cell mass compared to the untreated GK group; this effect resulted from both differentiation (neogenesis) and proliferation enhancement of β-cells. Follow up from day seven to the adult age (two months) showed that both treatments decreased postabsorptive basal plasma glucose levels and increased pancreatic insulin content compared to the untreated GK arm. In GK/GLP-1 and GK/exendin-4 groups, β-cell mass was significantly increased and represented 71% and 63% of the β-cell mass of the Wistar group, respectively. Glucose-stimulated insulin release, as evaluated during an IVGTT, was significantly improved in both treated groups. It was concluded that GLP-1 or exendin-4 treatment limited the prediabetic period and delayed the development of T2D in this animal model of prediabetes.

Exendin-4 activity was explored in a rat model of uteroplacental insufficiency[72]. Intrauterine growth retarded (IUGR) rats experience a progressive decline in β-cell mass weeks before the onset of T2D; hence there is a prediabetic neonatal period, which was investigated. At two weeks, exendin-4 significantly decreased body weight in both IUGR and control pups and this effect persisted into adulthood. It also improved glucose tolerance, which was maintained at 7 wk of age. Interestingly, at three months of age, vehicle-treated IUGR rats developed T2D (their β-cell mass declined by almost 80%) whereas exendin-4 treated IUGR rats had NGT and normal β-cell mass. At 18 months of age, exendin-4 treated IUGR rats were normoglycemic, while all vehicle treated IUGR rats had died. Exendin-4 therapy in IUGR rats at 14 d restored PDX-1 mRNA levels, in concentrations similar to controls; this effect persisted for three months.

Clinical studies: One hundred fifty two obese [average body mass index (BMI): 39.6 ± 7.0 kg/m^2] individuals with NGT or IGT or IFG were randomized to receive either exenatide (n = 73) (10 μg with a 4-wk 5 μg dose titration period) or placebo (n = 79), along with lifestyle modification for 24 wk[73]. Thirty eight individuals (25%) had IFG or IGT. Exenatide-treated individuals lost 5.1 ± 0.5 kg from baseline *vs* 1.6 ± 0.5 kg in the placebo group (treatment difference: -3.3%, P < 0.001). An important percentage of individuals with prediabetes returned to NGT after the end of the period (77% compared to 56% in the placebo group). No significant baseline to end point changes was shown for FPG, A1C and OGTT. Diarrhea was reported by 14% and 3% and nausea by 25% and 4% of the exenatide and placebo groups, respectively. Adverse effects were mild or moderate in severity in most cases. It was concluded that exenatide therapy in addition to lifestyle modification is a promising therapeutic approach for obese prediabetic individuals.

In another non randomized study, 105 individuals with IGT and/or IFG were treated with: (1) Lifestyle modification only (n = 18). Participants were advised to achieve 7% body weight loss over three months and to walk 30 min daily, seven days per week; (2) Pioglitazone 15mg daily and metformin 850mg daily (n = 40); and (3) A triple combination of pioglitazone 15mg daily, metformin 850 mg daily and exenatide 10 mcg twice daily (n = 47)[74]. All individuals who received drug therapy had the same advice on lifestyle intervention. Mean follow-up period was 8.9, 6.9, and 5.5 mo in the three groups respectively. Individuals in the lifestyle intervention group achieved only a slight reduction of body weight (82.3 kg to 80.9 kg). No significant change on insulin sensitivity and β-cell function was observed. In the pioglitazone and metformin group FPG was decreased from 109 mg/dL to 102 mg/dL and mean glucose AUC during OGTT was reduced by 12% (P < 0.001). Insulin sensitivity and β-cell function improved by 42% and 50% respectively, while 14% of the individuals with IGT and 36% of the individuals with IFG reverted to NGT. Interestingly, in the triple therapy group, a robust 109% improvement in β-cell function and a 52% increased in insulin sensitivity was observed, while 59% of the individuals with IGT and 56% of the individuals with IFG reverted to NGT. No patient in both double and triple therapy groups developed T2D.

A 24-wk prospective randomized outpatient clinical trial explored the possible role of exenatide (10 μg twice daily) and metformin (1000 mg twice daily), alone or in combination, on menstrual cyclicity and metabolic and endocrinological parameters in 60 overweight/obese women with polycystic ovary syndrome (PCOS)[75]. Forty two participants (70%), 14 in each arm completed the study protocol. Weight loss was more profound in the exenatide arms compared to metformin (P = 0.003). Combination treatment promoted a dramatic improvement in central adiposity. At the end of the study, the combination arm experienced weight loss of 6 ± 0.5 kg, the exenatide arm 3.2 ± 0.1 kg, and the metformin arm 1.6 ± 0.2 kg. Eighteen women with PCOS had glucose intolerance and 11 of them completed the study. Seven (64%) of them had NGT at the end of the trial (three of three in the combination arm, three of five on the metformin arm and one of three on the exenatide arm). Insulin sensitivity and HOMA-IR were significantly improved in all treatment groups. Insulin secretion, as measured by the corrected insulin response at glucose peak, was significantly reduced in the exenatide and combination arms (P < 0.016). The insulin secretion-sensitivity index increased progressively from metformin arm (232 ± 116) to the exenatide arm (395 ± 112) and the combination arm (516 ± 117) (P < 0.005), suggesting an improved β-cell function with enhanced insulin sensitivity.

The role of exenatide in order to improve postprandial endothelial function in individuals with IGT (n =

16) and patients with recent T2D with optimal glycemic control (n = 12) was investigated in a double-blinded randomized crossover study[76]. Endothelial function was estimated by reactive hyperemia peripheral arterial tonometry (PAT). In individuals with IGT, PAT index tended to increase after exenatide and was higher compared to the placebo period. Exenatide reduced postprandial rises in insulin, glucose and triglycerides concentrations. Postprandial PAT index was inversely correlated only with mean postprandial concentrations of triglycerides, possibly due to the high fat content of the meal administered. Change in postprandial triglycerides after exenatide accounted for 64% of the estimated effect of exenatide on postprandial endothelial function. Exenatide also reduced the postprandial elevation of triglycerides, apolipoprotein B-48, apolipoprotein CⅢ, remnant lipoprotein cholesterol and remnant lipoprotein triglyceride in individuals with IGT (n = 20) and patients with recent onset T2D (n = 15)[77]. These effects were not affected either with statin therapy or by glucose tolerance status. Both studies suggested an additional cardiovascular benefit of this agent beyond the improved glycemic control in this population[76,77]. Another randomized 3-wk head-to-head study examined the effects of exenatide *vs* metformin on microvascular endothelial function in 50 individuals with abdominal obesity and prediabetes[78]. Similar effects of both agents were shown on microvascular endothelial function, vascular activation, oxidative stress and markers inflammation. Exenatide did not demonstrate any beneficial effect on postprandial function in individuals with IGT. It was suggested that the reason for this observation was the administration of a glucose-only meal instead of a high fat meal, which would be expected to increase postprandial triglycerides[76,78].

Liraglutide

Studies organized in animal models: Liraglutide is a long acting analog with 97% homology to human GLP-1. It has an additional 16-carbon fatty acid and a small amino acid-spacer that promotes reversible binding to albumin and enhances resistance to DPP-Ⅳ degradation, providing a half-life of approximately 13 h[79]. The possible role of chronic liraglutide therapy in prediabetic UCD-T2D rats, in order to prevent or delay T2D, was investigated in a well organized study[80]. The UCD-T2D rat model develops polygenic adult-onset obesity and insulin resistance, followed by inadequate β-cell compensation and eventually T2D. UCD-T2D rats develop diabetes in a later age than other animal models of T2D; thus they are highly suitable for diabetes prevention studies[81]. At two months of age male sibling rats were divided in three groups (n = 32 per group): a control group (higher energy intake, body weight and adiposity compared to the other groups), a food-restricted group and a liraglutide group (0.2 mg/kg sc for 15 mo). Restricted rats were food restricted to 9% less energy per kg of body weight compared to the liraglutide group, in order to equalize body weights between these two groups. Half of the animals in each group were killed at 6.5 mo for tissue collection, while the remaining half continued treatment until T2D onset. FPG and A1C were lower in the liraglutide and food-restricted groups. Liraglutide treatment delayed T2D onset by 4.1 ± 0.8 mo compared to controls (P < 0.0001) and by 1.3 ± 0.8 mo compared to restricted animals (P < 0.05). Liraglutide-treated animals had lower fasting plasma triglycerides, glucagon and leptin levels, as well as body fat (despite similar body weight), compared to both groups. Decreased body fat could be the result of an increased lipid oxidation. Rats in the liraglutide group had significantly lower fasting plasma insulin compared to the other groups (P < 0.001), starting from one month and lasting throughout the 6 mo period, suggesting that this effect was not solely related to reduced body weight. Liraglutide treatment and energy restriction equally preserved pancreatic insulin content and islet morphology, possibly due to the lower weight gain and delayed hyperglycemia. Pancreatic insulin content in the control group was approximately one-third of that of the two other groups.

In another study, 12-wk old Otsuka-Long-Evans-Tokushima fatty (OLETF) rats (n = 8) were treated with three doses of liraglutide (50, 100, and 200 μg/kg twice a day) or 0.9% saline intraperitoneally (n = 8), twice daily for 12 wk. Eight Long-Evans-Tokushima-Otsuka rats with saline injection served as normal controls[82]. At the end of the 12 wk of treatment, all rats were euthanized and pancreatic tissues were used for histopathological and immunohistochemical analysis; only in the liraglutide 100 μg/kg group an analysis was performed, since this dose can be converted to a human equivalent dose. OLETF rats experienced obesity, IFG, hyperinsulinemia, insulin resistance, increased cholesterol levels, and a high inflammatory state. Although liraglutide treatment had only an acute effect on food intake, its beneficial effect on weight loss was sustained independently of feeding. All three doses of liraglutide suppressed IFG, IGT and insulin resistance. At the end of the 12-wk intervention period, 87.5% of the vehicle-treated OLETF progressed to T2D. On the contrary, 42.9% of IFG rats were reversed to NGT, while none of the liraglutide-treated OLETF rats progressed to T2D compared to vehicle-treated animals (P < 0.0001). Liraglutide improved both triglyceridemia and the inflammatory state observed. It also preserved islet morphology. Up-regulation of the anti-apoptotic Bcl-2 protein and down-regulation of the pro-apoptotic Bax factor were reported, which may contribute to the improvement of pancreatic islet function and structure.

When liraglutide was administered in a dose of 150 mg/kg twice daily for 6 wk in prediabetic rats, it strongly attenuated T2D development[83]. Approximately 53% of the antihyperglycemic effect observed was mediated by a reduction in food intake. In the experiments with 60% pancreatectomized rats, liraglutide significantly reduced glucose excursions after an OGTT. Furthermore, when NGT status was established, no increase in β-cell proliferation and mass was observed in both models of

β-cell deficiencies. It was suggested that the influence of GLP-1 agonism on β-cell mass dynamics *in vivo* was strongly related to the glycemic state observed.

Clinical studies: In a 20-wk prospective multicentre study, 564 nondiabetic obese individuals (31% of whom had prediabetes) were randomized to receive either one of four doses of liraglutide (1.2 mg, 1.8 mg, 2.4 mg, or 3.0 mg, *n:* 95, 90, 93 and 93, respectively) or placebo (n = 98) administered once daily subcutaneously or open label orlistat 120 mg three times daily (n = 95)[84]. All individuals increased their physical activity using pedometers and were advised to adhere a low fat diet with about to 500 kcal per day deficit. Sixty-one percent of the individuals in the liraglutide groups lost at least 5% of body weight from baseline, which was significantly more than the placebo arm. The proportion of individuals who lost more than 10% of baseline weight was dose depended and was greater in the 3 mg liraglutide arm than in the placebo arm (28% *vs* 2%). Systolic/diastolic blood pressure was reduced by 5.7/3.7 mmHg. The incidence of metabolic syndrome was reduced by more than 60% in those treated with liraglutide 2.4 mg and 3.0 mg. The prevalence of prediabetes was decreased by 84-96% with liraglutide 1.8 mg, 2.4 mg and 3 mg. Mean FPG was decreased by 7%-8% in the liraglutide arm, while no visible effect was described in the two other arms. Mean A1C was slightly reduced in a dose depended fashion in individuals treated with liraglutide compared to that in the two other groups. Mean change in plasma glucose during OGTT was reduced in all liraglutide groups compared to that of orlistat and placebo. Liraglutide therapy did not have any effect on insulin resistance as estimated by HOMA. However, median β-cell function was decreased with orlistat and placebo by 21% and 17% respectively, but increased in the liraglutide arm by 5%-24%. Fasting insulin levels initially increased, but as body weight and glucose concentrations gradually decreased, insulin levels were reduced, suggesting the glucose-depended activity of liraglutide on insulin secretion.

The two-year results from the extension of this 20-wk trial were recently reported[85]. Three hundred ninety eight individuals entered the extension and 268 (67%) completed the two-year trial. All participants continued on randomization treatment for one year, after which liraglutide or placebo individuals switched initially to liraglutide 2.4 mg and then 3 mg (based on 20-wk and one-year results, respectively). After two years, individuals on liraglutide 2.4/3.0 mg lost 3.0 kg (1.3-4.7 kg) more weight than those on orlistat ($P < 0.001$). Approximately 70% of the individuals on liraglutide 2.4/3.0 mg maintained weight loss more than 5% of screening weight after two years, 43% maintained more than 10% loss and 25% maintained more than 15% loss. Estimated weight loss of 7.8 kg and mean systolic blood pressure reduction of 12.5 mmHg was sustained with liraglutide 2.4/3.0 mg in completers from screening. Between 52%-62% of liraglutide-treated individuals with prediabetes at randomization achieved NGT after two years compared to 26% in the orlistat arm. Mean FPG and A1C concentrations were also reduced. The two year prevalence of prediabetes and metabolic syndrome in the liraglutide 2.4/3.0 mg group was decreased by 52% and 59% respectively. The most frequent liraglutide-associated adverse effects were gastrointestinal, mainly nausea and vomiting, as expected from T2D trials. However, most nausea/vomiting episodes were transient; more than 90% were mild or moderate in intensity.

Recently, a 14-wk double blind, randomized placebo-controlled study was launched in order to investigate the possible role of liraglutide 1.8 mg treatment in 68 older (mean age: 58 ± 8 years) overweight/obese (mean BMI: 31.9 kg/m^2) individuals with prediabetes (IFG and/or IGT)[86]. Participants were also advised to eat a moderate carbohydrate diet and decrease total caloric intake by 500 kcal/d. Twenty four (68%) individuals randomized in the liraglutide group and 27 (82%) individuals in the placebo group completed testing at the end of the trial. Participants randomized to liraglutide arm lost twice as much weight as those assigned to placebo (6.8 kg *vs* 3.3 kg; $P < 0.001$). More individuals in the liraglutide arm finally lost 7% of baseline weight compared to the placebo arm (54% *vs* 4%); 10% weight loss was only observed in the liraglutide arm (17%). Weight loss after liraglutide therapy was associated with significant reduction of insulin resistance. Steady state plasma glucose concentrations were reduced by 29% in the liraglutide arm compared with no change in the placebo arm; FPG (-0.5 mmol/L *vs* 0 mmol/L), systolic blood pressure (-8.1 mmHg *vs* -2.6 mmHg), and triglyceride levels (-0.4 mmol/L *vs* -0.1 mmol/L) were also significantly decreased in the liraglutide arm compared to the placebo arm respectively ($P \leqslant 0.04$). In addition, 75% of the participants in the liraglutide arm achieved normal FPG. The most common adverse effect in the liraglutide arm was nausea (67% *vs* 26% in the placebo arm). It was suggested that the improvement of glycemia in the liraglutide group appeared to be better than reported with weight loss alone in this population.

Indeed, the effects of GLP-1R agonists on insulin secretion are not a simple phenomenon. These medications can increase glucose secretion in a glucose-depended manner after acting directly on the β-cell; they can also decrease insulin secretion secondary to weight loss and enhancement of insulin sensitivity. In this view, it is unclear what the net effect would be when they are administered in individuals with prediabetes. In order to investigate this observation, a parallel study was organized in order to evaluate the relative impact of the indirect effect of weight loss and increase insulin sensitivity compared to the direct effect of GLP-1R agonists on β-cell function[86,87]. In this recent double-blind, randomized, placebo-controlled, parallel-group study 49 individuals (mean age: 58 years, mean BMI: 32.9 kg/m^2) with prediabetes (isolated IFG, isolated IGT and combined IFG/IGT) received either liraglutide 1.8 mg daily (n = 24) or placebo (n = 25). All participants were instructed

to decrease total energy intake by 500 kcal per day and to continue their baseline physical activity[87]. There was a little overlap in the degree of weight loss between the two arms since 88% of the individuals in the liraglutide arm lost more than 5% of baseline body weight compared to 22% in the placebo arm. Weight loss promoted a significant improvement on insulin resistance in the liraglutide arm compared to the placebo arm (-7.7% *vs* -3.9%, $P < 0.001$). Insulin response, after intravenous glucose infusion, was decreased by 7% in the placebo arm whereas it increased by 34% in the liraglutide arm. C-peptide AUC was increased by 29% in individuals receiving liraglutide and NEFAs concentration was reduced. Placebo treatment had no effect on these two parameters. Regression analyses suggested that weight loss was not associated with any changes in pancreatic β-cell function. Despite weight loss and reduction of insulin resistance in the liraglutide arm, the insulin secretion rate was significantly increased and there was no association between weight loss and changes on insulin secretion. It was concluded that changes following liraglutide treatment in patients with prediabetes are not those that are described after weight loss and improved insulin sensitivity, but rather similar effects after an acute GLP-1 infusion[87,88].

SAFETY OF INCRETIN-BASED THERAPIES

An acceptable safety profile is of major importance for every intervention administered in order to prevent or delay T2D. As far as GLP-1R agonists are concerned, the most common adverse effects are gastrointestinal, including nausea, vomiting and diarrhea[89]. However, they occur early on during treatment and tend to be transient. For DPP-4 inhibitors, adverse effects resemble that of placebo, with nasopharyngitis and headache being the most common described[90]. Moreover, discontinuation of therapy because of side effects was similar to placebo[91].

Small preclinical studies, as well as some post-marketing reports, raised the possibility of an increased risk of pancreatitis with incretin based therapies[92-96]. In a study that data were collected from the Food and Drug Administration (FDA) adverse event reporting system database, GLP-1 based therapies were associated with pancreatitis and pancreatic cancer[97]. Another case-control study reported an increased risk for hospitalization for acute pancreatitis with GLP-1 based therapies (after combining exenatide and sitaglitpin treatments) and adjusting for potential confounders[98]. Concerns were also raised after the results of a study organized in organ donors with T2D, who received either sitagliptin or exenatide. A possible expansion of endocrine and exocrine pancreatic compartments after incretin-based therapy, the former being associated by α-cell hyperplasia with the potential progression to neuroendocrine tumors and the latter with an enhanced proliferation and dysplasia, was described[99]. Furthermore, a recent case-control analysis, based on the French pharmacovigilance database, suggested an association of all incretin-based therapies with pancreatitis[100]. A trend towards a slightly elevated risk of pancreatitis, only with GLP-1R agonists, was also shown in a recent pooled analysis of phase Ⅲ trials, although the number of cases was very small and the statistical power was limited[101].

However larger preclinical studies did not established an association of incretin-based therapies with pancreatitis[102-109]. Interestingly in three of these studies, GLP-1R activation or DPP-4 inhibition had a beneficial effect on exocrine pancreatic function and structure[103,104]. A recent study also suggested that pancreatic findings attributed to incretin-based therapies in rodents are commonly observed background findings, without any drug treatment and independent of diet or glycemic status[110]. Moreover large retrospective population studies and recent meta-analysis suggested a negative association of incretin-based therapies with either pancreatitis or pancreatic cancer[111-120]. Recently the FDA reevaluated more than 250 toxicology studies, organized in nearly 18000 healthy animals, and found no association with pancreatitis or any pancreatic toxicity. The European Medicines Agency conducted a same review and reported no pancreatic tumors in mice and rats treated with incretin-based drugs, even at doses that greatly exceed the level of human clinical exposure[121].

A higher expression of GLP-1Rs in rodent calcitonin-producing thyroid C cells, (mainly in rats and mice) combined with sustained GLP-1R activation can result in stimulation of calcitonin secretion, hyperplasia, adenoma and eventually medullary thyroid cancer[122,123]. Indeed, both liraglutide and exenatide were shown to promote the development of thyroid C cell cancer after chronic therapy in rodents[122]. An elevated risk for thyroid carcinoma was described in one study[97]. However, thyroid C cells in humans and monkeys express lower levels of GLP-1Rs[124]. Long-term treatment with high doses liraglutide did not produced thyroid C cell proliferation in monkeys, while no association between calcitonin levels and liraglutide, up to 3 mg daily, was established in large numbers of patients with T2D[125].

Retrospective analysis of phase Ⅲ clinical trials, in which major cardiovascular events were reported as adverse events, have been published for exenatide, liraglutide, vildagliptin, sitagliptin, alogliptin, saxagliptin, and linagliptin[126]. In all of these studies the relative risk for a major cardiovascular event (acute myocardial infarction, stroke and cardiovascular death) was reduced relative to placebo or a comparator therapy to a value below one. However, the 95%CI was more than one in most of these studies, thus the number of events was too small so as to extract definite conclusions. Both the Saxagliptin Assessment of Vascular Outcomes Recorded (SAVOR)-Thrombolysis in Myocardial Infarction (TIMI) 53 (SAVOR-TIMI 53) and the Examination of Cardiovascular Outcomes with Alogliptin *vs* Standard of Care (EXAMINE) trials met the FDA criteria for non inferiority of saxagliptin and alogliptin over placebo respectively, but unfortunately they did not demonstrated any positive evi-

Table 1 Main clinical studies of dipeptidyl peptidase-4 inhibitors in a prediabetic state

Ref.	Study population	Study design	Main results
Utzschneider *et al*[50]	22 individuals with IFG	VILDA was administered in a dose of 100 mg daily for 6 wk. Two weeks of placebo treatment before (running period) and after (washout period) 6 wk was studied	FPG levels were not significantly reduced. AUC GLU and 2-h GLU decreased after a MTT. DI was increased by 69% and insulin sensitivity by 25% after an IVGTT These effects were not sustained in the washout period
Rosenstock *et al*[51]	179 individuals with IGT (80%: IFG + IGT)	Multicenter 12-wk double-blind study 90 participants received VILDA 50 mg/daily and 89 received placebo therapy	Improvements in β-cell function as estimated by insulin secretion relative to that of GLU. Improvements were also reported in α-cell function. These beneficial effects contributed to approximately 30% reduction in prandial GLU excursions
Werzowa *et al*[52]	48 IGT renal transplant recipients	3-mo, double-blind, placebo-controlled study. Participants were randomized to receive 50 mg of VILDA, 30 mg of PIO or placebo in a 1:1:1 ratio (n = 16 in each arm)	A1C reduction was statistically significant between treatment groups and placebo. VILDA and PIO reduced the 2 h plasma GLU at three months compared with baseline, while only PIO reduced FPG
Bock *et al*[57]	22 individuals with IFG	8-wk double blind placebo-controlled study Participants received SITA 100 mg daily (n = 11) or placebo (n = 11)	SITA increased postprandial intact GLP-1 concentrations. Both fasting and postprandial GLU values were unchanged with SITA therapy. A slightly increased DI was reported
Perreault *et al*[58]	23 individuals with either IFG (n = 10) or NGT (n = 13)	4-wk open-label, parallel group study. All participants received SITA 100 mg once daily	SITA resulted in a small, but significant decrease in FPG compared to baseline in both groups (P < 0.05) Administration of SITA did not altered insulin or GLU excursions in the post-intervention OGTT, but did increase AUC for active GLP-1 and C-peptide compared to baseline levels (P < 0.01 for both)

GLP-1: Glucagon-like peptide 1; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance; NGT: Normal glucose tolerance; FPG: Fasting plasma glucose; AUC: Area under the curve; DI: Disposition index; IVGTT: Intravenous glucose tolerance test; MTT: Meal tolerance test; A1C: Glycated hemoglobulin; VILDA: Vildagliptin; SITA: Sitagliptin; PIO: Pioglitazone; GLU: Glucose; OGTT: Oral glucose tolerance test.

dence on cardiovascular risk reduction[127,128]. Two recent meta-analysis suggested that DPP-4 inhibitors may have a neutral effect or reduce the risk of cardiovascular events and all-cause mortality in patients with T2D[129,130]. As far as GLP-1R agonists are concerned two recent meta-analysis reported that these agents do not appear to increase cardiovascular morbidity in comparison with placebo or other active drugs[131,132].

Hospitalization for heart failure among T2D who received saxagliptin in the SAVOR-TIMI 53 was increased by 27% compared to the placebo group (3.5% *vs* 2.8%; HR = 1.27; 95%CI: 1.07-1.51; P = 0.007), while no association of alogliptin with heart failure was found in the EXAMINE study[133]. Two recent meta-analysis suggested a possible increased risk of developing heart failure after DPP-4 therapy[134,135]. Currently, a large number of long-term cardiovascular outcome trials in patients with T2D are being performed in order to clarify the cardiovascular safety and efficacy of incretin-based therapies[136].

In addition to safety and efficacy of incretin-based therapies, cost is another significant issue that must be taken into consideration. Although the cost of incretin-based therapies is greater compared to other glucose-lowering therapies, long term effectiveness of these agents can be associated with a decreased in the cost of management of T2D and its complications compared to other therapies[137].

CONCLUSIONS-PERSPECTIVES

During the last two decades there has been an immense investigation in order to understand the pathophysiology of the early stages of hyperglycemia, which very often progress to overt T2D within a few years, as β-cell decline and failure progresses. The huge burden resulting from the complications of T2D created the need of novel therapeutic strategies in an effort to prevent its development[8]. The beneficial effects of incretin-based therapies on β-cell function in patients with T2D, together with their strictly glucose-depended mechanism of action, suggested their possible use in individuals with prediabetes, when greater β-cell mass and function are preserved and the possibility of β-cell salvage is higher[138]. The main results of the most important clinical studies of incretin-based therapies in individuals with prediabetes are shown in Tables 1 and 2.

DPP-4 inhibitors have shown beneficial effects on β-cell mass and function in preclinical models of prediabetes. However short-term clinical studies (maximum duration of 12 wk) have only demonstrated a modest effect on glucose homeostasis, which was lost after treatment discontinuation[50]. Whether longer periods of DPP-4 inhibition in individuals with prediabetes can measurably alter β-cell function, in a way that is sustained even after treatment discontinuation, remains unproven. One year treatment with vildagliptin in drug-naïve patients with T2D and mild hyperglycemia initially increased β-cell secretory capacity, but this effect was not maintained after the washout period[139]. However, when vildagliptin was administered in drug-naïve patients with T2D and mild hyperglycemia (A1C: 6.2%-7.2%) for two years, β-cell function tended to be greater after two years than after

Table 2 Main clinical studies of glucagon-like peptide-1 receptor agonists in a prediabetic state

Ref.	Study population	Study design	Main results
Rosenstock *et al*[73]	152 obese individuals of whom 38 had IGT or IFG	Participants were randomized to receive either EXE (n = 73) (10 μg with a 4-wk 5 μg dose titration period) or placebo (n = 79) along with lifestyle modification for 24 wk	EXE-treated individuals lost 5.1 ± 0.5 kg from baseline *vs* 1.6 ± 0.5 kg in the placebo group (P < 0.001). An important percentage of individuals with prediabetes returned to NGT after the end period (77% compared to 56% in the placebo group)
Armato *et al*[74]	105 individuals with IGT and/or IFG. Mean follow-up period was 8.9, 6.9, and 5.5 mo in the three groups respectively	Participants were treated with: (1) Lifestyle modification only (n = 18); (2) PIO 15 mg daily and MET 850 mg daily (n = 40); and (3) PIO 15 mg daily, MET 850 mg daily and EXE 10 mcg twice daily (n = 47)	A robust 109% improvement in β-cell function and 52% increased in insulin sensitivity was observed in the EXE group, while 59% of individuals with IGT and 56% individuals with IFG reverted to NGT. No patient in both double and triple therapy groups developed T2D
Astrup *et al*[84]	564 obese individuals (31% had prediabetes)	20 wk double-blind prospective multicentre study. Participants were randomized to receive either one of four doses of LIRA (1.2 mg, 1.8 mg, 2.4 mg, or 3.0 mg, n: 95, 90, 93 and 93) or placebo (n = 98) or open label orlistat 120 mg three times a day (n = 95)	61% of the individuals in the LIRA groups lost at least 5% of body weight from the baseline, which was significantly more than in the placebo arm. The prevalence of prediabetes was decreased by 84%-96% with LIRA 1.8 mg, 2.4 mg and 3 mg. Mean FPG was decreased by 7%-8% only in the LIRA arm. Mean change in plasma GLU during OGTT were reduced in all LIRA groups compared with that of orlistat and placebo. Median β-cell function increased in the LIRA arm by 5%-24%
Kim *et al*[86]	68 overweight/obese individuals with IFG and/or IGT	14 wk double blind randomized placebo-controlled study. 24 individuals received LIRA 1.8 mg daily and 27 placebo therapy	Participants randomized to LIRA arm lost twice as much weight as those assigned to placebo (P < 0.001). Steady sate plasma GLU was reduced by 29% in the LIRA arm compared with no change in the placebo arm. 75% of the participants in the LIRA arm achieved normal FPG
Kim *et al*[87]	49 individual with isolated IFG, isolated IGT and combined IFG/IGT	14 wk double-blind, randomized, placebo-controlled, parallel-group study. Participants received LIRA 1.8 mg daily (n = 24) or placebo (n = 25)	Weight loss promoted a significant improvement in insulin resistance in the LIRA arm compared to the placebo arm (-7.7% *vs* -3.9%, P < 0.001). Insulin response, after intravenous GLU infusion, was decreased by 7% in the placebo arm whereas it increased by 34% in the LIRA arm. Despite weight loss and reduction of insulin resistance in the LIRA arm, the insulin secretion rate was significantly increased and there was no association between weight loss and changes in insulin secretion

IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance; NGT: Normal glucose tolerance; FPG: Fasting plasma glucose; EXE: Exenatide; LIRA: Liraglutide; PIO: Pioglitazone; MET: Metformin; T2D: Type 2 diabetes; GLU: Glucose; OGTT: Oral glucose tolerance test.

one year of treatment[140].

GLP-1R agonists have also shown significant improvements on β-cell mass and function in preclinical studies. Important improvements on β-cell function and insulin sensitivity were also reported in short term clinical studies, in which an important percentage of individuals with prediabetes returned to NGT. Weigh reduction in overweight and obese individuals with prediabetes was also shown, as well as improvements of endothelial function and lipid profile. Whether GLP-1R agonists can prevent or delay the transition to T2D needs further investigation in well-designed long term studies. The Restoring Insulin Secretion consortium will examine whether medication, including liraglutide, or surgical intervention strategies can reduce the progressive β-cell dysfunction in adults and youth with prediabetes or early T2D[141]. The duration of GLP-1R agonists therapy in order to promote sustained β-cell improvements is also an issue of investigation. Interestingly, when exenatide was administered in patients with T2D for one year, the treatment related improvement of β-cell function was lost after a four-week drug cessation[142]. However, the three-year data of exenatide treatment suggested a small but statistically significant effect on DI following a four-week off therapy period[143].

Recent evidence also demonstrates the presence of genetically induced GLP-1 resistance both in prediabetic and diabetic states. Whether pharmacogenomic studies are needed in order to identify responders and non-responders to incretin based therapies regarding glucose metabolism, is an issue of future research[144].

The safety of incretin-based therapies remains a topic of scientific discussion and exploration[126,145,146]. Currently, precise estimates for the risk of possible serious adverse effects associated with incretin-based therapies cannot be estimated. Future data from cardiovascular outcome studies and ongoing clinical studies, which will improve the statistical power of prospective studies and facilitate larger meta-analyses, are crucially anticipated in order to clarify their long-term safety. Until these data are available, large, long term, well designed future diabetes prevention trials of incretin-based therapies will be required in order to determine whether they can stabilize or reverse β-cell loss and promote a sustained reduction in the development of T2D in this population.

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REVIEW

Risks of rapid decline renal function in patients with type 2 diabetes

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Abstract

Progressive rising population of diabetes and related nephropathy, namely, diabetic kidney disease and associated end stage renal disease has become a major global public health issue. Results of observational studies indicate that most diabetic kidney disease progresses over decades; however, certain diabetes patients display a rapid decline in renal function, which may lead to renal failure within months. Although the definition of rapid renal function decline remained speculative, in general, it is defined by the decrease of estimated glomerular filtration rate (eGFR) in absolute rate of loss or percent change. Based on the Kidney Disease: Improving Global Outcomes 2012 clinical practice guidelines, a rapid decline in renal function is defined as a sustained decline in eGFR of > 5 mL/min per 1.73 m^2 per year. It has been reported that potential factors contributing to a rapid decline in renal function include ethnic/genetic and demographic causes, smoking habits, increased glycated hemoglobin levels, obesity, albuminuria, anemia, low serum magnesium levels, high serum phosphate levels, vitamin D deficiency, elevated systolic blood pressure, pulse pressure, brachial-ankle pulse wave velocity values, retinopathy, and cardiac autonomic neuropathy. This article reviews current literatures in this area and provides insight on the early detection of diabetic subjects who are at risk of a rapid decline in renal function in order to develop a more aggressive approach to renal and cardiovascular protection.

Key words: Type 2 diabetes; Diabetic kidney disease; Rapid decline; Estimated glomerular filtration rate; Albuminuria

Core tip: The progression rate of diabetic kidney disease is highly variable, a rapid decline of renal function can lead to renal failure within months. Risk factors account for rapid decline renal function in patients with type 2 diabetes include ethnic/genetic and demographic factors, lifestyle and health behaviors, advanced albuminuria, poor glycemic control, dyslipidemia and some biochemical abnormalities. Diabetic patients with retinopathy or cardiac autonomic neuropathy are at increased risk of a rapid decline in estimated glomerular filtration rate. Early detection of high-risk groups with a more aggressive multifactorial approach to renal and cardiovascular protection is important.

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INTRODUCTION

Type 2 diabetes is one of the leading causes of chronic kidney disease (CKD) worldwide, and diabetic kidney disease has become a major global public health issue[1]. Early detection and intervention in diabetic kidney disease can help to slow renal function decline, prevent complications, and decrease cardiovascular events, thereby improving survival and quality of life in type 2 diabetics[2]. However, potential causes accounting for variation in diabetic kidney disease and its rate of progression are still largely unexplored. In most cases, disease progresses over decades; however, a rapid decline in renal function can lead to renal failure within months[3]. Thus, in type 2 diabetics, defining high-risk groups and preventing or retarding disease progression is an emerging challenge. This review targets the potential risk factors of a rapid decline in renal function in patients with type 2 diabetes.

EPIDEMIOLOGY OF DIABETIC KIDNEY DISEASE

Diabetic kidney disease is identified clinically through the presence of albuminuria, impaired glomerular filtration rate (GFR), or both[4], and these two biomarkers have been used for the diagnosis, severity classification, and outcome prediction of CKD[5-8]. The categories of albuminuria are defined as microalbuminuria or macroalbuminuria based on a urinary albumin-to-creatinine ratio (UACR) of 30-300 mg/g, or > 300 mg/g, respectively[9,10], and impaired renal function is defined as an estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m^2[1,4,10]. International consensus on the incidence of CKD in patients with type 2 diabetes is lacking[11]. Although the prevalence of diabetic CKD is increasing worldwide, there are large differences between regions and ethnicities (Table 1). A report from the UK Prospective Diabetes Study (UKPDS), states that 1544 (38%) of 4031 patients developed albuminuria (microalbuminuria or macroalbuminuria), and 1449 (29%) of 5,032 patients developed renal impairment (based on the Cockroft-Gault formula of eGFR < 60 mL/min per 1.73 m^2) over a 15-year period[12]. Meanwhile, the Developing Education on Microalbuminuria for Awareness of renal and cardiovascular risk in Diabetes (DEMAND) study, in which data from 32208 type 2 diabetics from 33 countries were collected, reported that overall global prevalence of microalbuminuria and macroalbuminuria was 39% and 10% respectively, while eGFR below 60 mL/min per 1.73 m^2 occurred in 22% of the 11573 patients with available data[13]. According to the US Renal Data System (USRDS) 2013 report, 3 out of 5 new end stage renal disease (ESRD) patients came from diabetes in Malaysia, Mexico, and Singapore; furthermore in the United States, the odds ratios of diabetes in albuminuria (UACR more than 30 mg/g) and CKD (defined as eGFR below 60 mL/min per 1.73 m^2) were 3.9 and 2.1 respectively[14]. It was recently reported that 30% of CKD in 5584 Chinese patients aged 20-79 years, was associated with dysglycemia (diabetes and prediabetes), independent of age, sex, and hypertension status[15]. It should be noted that some limitations and pitfalls were identified in these epidemiological data, for example, demographic distribution[11], socioeconomic status[16], dynamic changes in the incidence of diabetes, changes in the use of medication (including anti-diabetic drugs and anti-hypertensive drugs), and the improvement of survival rates in diabetic and ESRD patients[11].

DEFINING A RAPID DECLINE IN RENAL FUNCTION

Annual decline in GFR in an individual varies widely depending on race, age, the presence of underlying conditions, the etiology of CKD, and the presence of comorbidities. A previous study reported that age-related eGFR decline is about 0.75-1 mL/min per 1.73 m^2 per year over 40 years of age[17]. Among the healthy population, eGFR decline is approximately 0.36-1.21 mL/min per 1.73 m^2 per year[5,18-21]. A community-based cohort study reported a decline in eGFR of 2.1 and 2.7 mL/min per 1.73 m^2 per year respectively for women and men with diabetes, whereas the rate of decline was 0.8 and 1.4 mL/min per 1.73 m^2 per year respectively for women and men without diabetes[18,22]. In subjects with CKD, a more rapid decline in renal function (ranging 1.03-4.3 mL/min per 1.73 m^2 per year) was noted[10,23-26] (Table 2). Some studies define rapid decline of eGFR in terms of absolute rate of loss, while others define it as percent change (Table 3)[3,27-30]. According to the Kidney Disease: Improving Global Outcomes (KDIGO) 2012 clinical practice guidelines for the evaluation and management of CKD, developed by the National Kidney Foundation, a rapid decline in renal function is defined as a sustained decline in eGFR of > 5 mL/min per 1.73 m^2 per year (as estimated using the 2009 CKD-EPI creatinine equation)[31]. It is generally believed that at present, there are a lack of well-controlled studies, which include frequent measurements and a long follow-up period, from which to establish an optimal definition of a rapid decline in renal function[18].

RISK FACTORS OF A RAPID DECLINE IN RENAL FUNCTION

An emerging challenge is the identification of potential factors associated with rapid renal function decline, which would form the basis for the development of strategies to prevent or retard disease progression, and reduce complications, thereby improving disease outcomes and quality of life in type 2 diabetics. Potential risk factors include ethnic/genetic and demographic factors, lifestyle and health behaviors, metabolic and biochemical abnormalities, cardiovascular functional factors, and some clinical symptoms of type 2 diabetes (Figure 1).

Table 1 Prevalence of albuminuria and impaired glomerular filtration rate in diabetic patients

Ref.	Population (Nationality)	Albuminuria prevalence	Impaired GFR prevalence
Parving *et al*[13]	International DEMAND study of 33 countries 2006 32208 type 2 diabetic patients	Microalbuminuria: 39% Macroalbuminuria: 10%	22%
Bos *et al*[108] data from: Herman *et al*[109]	Northern Africa Systematic review of PubMed 1990-2012	Egypt 1998: Albuminuria: 21%[109]	Egypt 1998-Outpatient clinics: 6.7%[109]
Hamed *et al*[111]	> 18 years old diabetic patients	Sudan 2008 (insulin treated diabetic patients): Albuminuria: 22%[110]	Egypt 1995-Hospital inpatients: 46.3%[111]
Icks A and Koch M Epidemiology of chronic kidney disease in diseases. In: Wolf G. Diabetes and Kidney Disease[11], data from: Chadban *et al*[112]	Australia AusDiab study: a national population-based cross-sectional survey > 25 years old diabetic patients	8.70% proteinuria-spot urine protein to creatinine ratio (abnormal: > 0.20 mg/mg)	27.60%
Unnikrishnan *et al*[113]	Southern India CURES 45 study 17, 16 type 2 diabetic patients	Microalbuminuria: 36.9% Macroalbuminuria: 2.2%	-
Icks A and Koch M Epidemiology of chronic kidney disease in diseases. In: Wolf G. Diabetes and Kidney Disease[11], data from: Lin *et al*[114]	Taiwan Community-based screening 1999–2001 > 30 years old type 2 diabetic patients	29.40% proteinuria-spot urine protein to creatinine ratio (abnormal: > 0.20 mg/mg)	15.10%
Yang *et al*[115]	China A nationally representative sample from 14 provinces and municipalities > 20 years old diabetic patients	17.30%	19.10%
Lou Arnal *et al*[116]	Spain A survey of 16 Health Centers of the Alcañiz Health Sector 2008 > 18 years old, 3466 type 2 diabetic patients	31.70%	25.20%
Detournay *et al*[117]	France ENTRED data 2007 A survey of the national public prescription claims database Type 2 diabetic patients	-	22%
Collins *et al*[14]	United Status NHANES study 2005-2010 Adult diabetic patients	29.90%	19.30%
Al-Rubeaan *et al*[54]	Saudi Arabia SNDR data > 25 yr, 54670 type 2 diabetic patients	Microalbuminuria: 1.2% Macroalbuminuria: 8.1%	GFR < 30 mL/min per 1.73 m^2: 1.50%

Albuminuria: Albumin-to-creatinine ratio (UACR) > 30 mg/g; Microalbuminuria: UACR 30-300 mg/g; Macroalbuminuria: UACR > 300 mg/g; Impaired glomerular filtration rate (GFR): Estimated GFR < 60 mL/min per 1.73 m^2; DEMAND: Developing Education on Microalbuminuria for Awareness of renal and cardiovascular risk in Diabetes study; AusDiab: The Australian Diabetes, Obesity and Lifestyle Study; CURES: Chennai Urban Rural Epidemiology Study; ENTRED: Échantillon national témoin représentatif des personnes diabétiques (National Representative Sample of Diabetic Patients); NHANES: National Health and Nutrition Examination Survey; SNDR: Saudi National Diabetes Registry.

Ethnic, genetic, and demographic factors

Ethnicity is a one of major factors affecting the progression of CKD in diabetic patients. In the United Kingdom, residents of South Asian origin had a higher prevalence of overt proteinuria and a lower prevalence of microalbuminuria compared to those with White European ethnicity[1,32]. In a 5-year retrospective, community-based cohort study of 135 general practices in East London, in which 3855 diabetic patients with an eGFR of < 60 mL/min per 1.73 m^2 were enrolled, renal function decline occurred at a significantly higher rate in South Asians as compared to other ethnicities[33]. According to the USRDS 2012 annual data report[34], ESRD caused by diabetes has increased in African-American, Native American, and Hispanic populations over the past decade[1,2,34]. USRDS 2013 also reported that the contribution of diabetes to ESRD was 59%-61% in Malaysia, Mexico, and Singapore in 2011, and above 40% in Israel, the Republic of Korea, Hong Kong, Taiwan, the Philippines, Japan, the United States, and New Zealand[14]. In summary, diabetic patients of Hispanic, black, Asian, and Maori ethnicity are at a higher risk of a rapid decline in renal function compared to white populations.

Ethnic differences in the presentation of diabetic kidney disease may reflect either genetic predisposition or differences in public health care policy[1], and thus, genetic studies need to exclude non-genetic confounders. Evidence of genes associated with diabetic nephropathy in type 2 diabetics comes mainly from family-based genome-wide linkage studies[35,36]. Findings from such studies include reports that 7p14.1 [engulfment and cell motility 1 (ELMO1)][37,38], 7q21.1/7q21.3[39] and 18q22.3

Table 2 Decline of estimated glomerular filtration rate in different populations

Population	eGFR decline (mL/min per 1.73 m^2 per year)	Ref.
Healthy		
PREVEND study 6894 subjects	0.55	Halbesma *et al*[5]
	Estimated using MDRD formula	
Annual health exam, Japan	0.36	Imai *et al*[19]
120727 subjects	Estimated using MDRD formula	
	modified by a Japanese coefficient	
ARIC study	0.47	Matsushita *et al*[20]
13029 subjects	Estimated using MDRD formula	
Tromso Study, Norway	1.21 (men)	Kronborg *et al*[21]
2249 men and 2192 women	1.19 (women)	
	Estimated using MDRD formula	
Aged without diabetes		
2475 men > 65 years old	1.4	Hemmelgarn *et al*[22]
3163 women > 65 years old	0.8	Hemmelgarn *et al*[22]
Aged with diabetes		
490 men > 65 years old	2.7	Hemmelgarn *et al*[22]
445 women > 65 years old	2.1	Hemmelgarn *et al*[22]
CKD		
MDRD study group	3.7	MDRD study group
eGFR 25-80 mL/min per 1.73 m^2, n = 28		Levey *et al*[23]
eGFR 7.5-24 mL/min per 1.73 m^2, n = 63	4.3	
African Americans with hypertension	2.21	Wright *et al*[24]
eGFR 20-65 mL/min per 1.73 m^2		
low mean arterial pressure, n = 380		
normal mean arterial pressure, n = 374	1.95	
Tromso Study, Norway	1.03	Eriksen *et al*[25]
eGFR 30-59 mL/min per 1.73 m^2		
3047 subjects		
eGFR < 60 mL/min per 1.73 m^2	2.65	Levin *et al*[26]
4231 subjects		

Data from Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group[18]; eGFR: Estimated glomerular filtration rate; CKD: Chronic kidney disease; PREVEND: Prevention of Renal and Vascular End-Stage Disease; ARIC: Atherosclerosis Risk in Communities; MDRD: Modification of Diet in Renal Disease Study.

[carnosine dipeptidase 1 (CNDP1)][40,41] are associated with the development of proteinuria and ESRD in African-Americans; 18q22.3 (CNDP1) is associated with proteinuria and ESRD in American-Indians[4]; and 17p14.1[37], 12q24.11 [acetyl-CoA carboxylase alpha (ACACB)][42], 13q34(rs1411766)[43], and 16q13 [solute-carrier group (SLC12A3)][44] may be associated with proteinuria and ESRD in Japanese[36]. Furthermore, haptoglobin (Hp) is a hemoglobin-binding protein that has a major role in protecting against heme-driven oxidative stress. Previous studies have shown the importance of the Hp genotype in the progression of diabetic nephropathy[45,46]. Moreover, diabetic patients with Hp 2-2 are more likely to develop nephropathy than those with Hp2-1 or Hp1-1[47,48].

Demographic factors may also influence the progression of diabetic kidney disease. Previous studies indicate that age is a significant predictor of progressive albuminuria and renal dysfunction in diabetics[49-54], and most studies reported that male sex is an important independent factor associated with renal function decline in type 2 diabetics[12,50,54,55]; however, some studies have shown an association with female sex[56].

Lifestyle and health behaviors

Smoking is an established factor for increased risk of development and rapid progression of diabetic kidney disease[12,54,57-59]. Also, some studies suggest an association between diet and renal function decline in diabetics, for example in those with high alcohol consumption[58] or a high-protein diet[59]. It has been demonstrated that a high dietary acid load (*e.g.*, in diets high in rice and meat) is associated with rapid progression of diabetic nephropathy to ESRD in Westernized South Asian people[60]. Lack of physical activity is also considered to be a risk factor in diabetic nephropathy[58], with a previous study reporting that high physical activity in women was associated with an improvement in eGFR[21].

Metabolic and biochemical factors

A number of metabolic conditions, such as hyperglycemia[61,62], dyslipidemia[63-65], or being overweight/obese[31,49,66], are widely recognized as being associated with the development of diabetic nephropathy, and are established factors in identifying subjects at a greater risk of disease progression[57]. Previous studies indicate that obesity, hyperglycemia, and dyslipidemia are significant predictors of progressive albuminuria[49-53,67,68]. A recent cross-sectional study reported UACR significantly correlated with metabolic syndrome and its components, including hyperglycemia, central obesity, and high triglyceride lev-

Table 3 Definitions of rapid renal function decline

	Population (Nationality)	Rapid renal function decline	Ref.
Study			
	United States 4380 patients from the community-based CHS ⩾ 65 years old Follow-up: 7 yr 14% with diabetes	> 3 mL/min per 1.73 m^2 per year	Reviewed by KDIGO CKD Work Group[18]: Shlipak *et al*[28] Rifkin *et al*[30]
	Taiwan 577 type 2 diabetes patients from an outpatient department in a hospital-based study 63 years old (mean age) Follow-up: 1 yr	> 3 mL/min per 1.73 m^2 per year	Sheen *et al*[72]
	472 CKD 4-5 patients from an outpatient department in a hospital-based study 65 years old (mean age) 35.4% with diabetes Follow-up: 1.5 yr (17.3 mo)		Tsai *et al*[118]
	Canada 4231 patients with eGFR < 30 mL/min per 1.73 m^2 from a cohort derived from all patients registered in a provincial database Follow-up: 2.5 yr (31 mo)	> 4 mL/min per 1.73 m^2 per year	Levin *et al*[26]
	Italy 1682 type 2 diabetes patients with eGFR ⩾ 60 mL/min per 1.73 m^2 from an outpatient department in a hospital based study Follow-up: 10 yr	> 4% per year	Zoppini *et al*[70]
	Canada 3154 patients with eGFR ⩾ 60 mL/min per 1.73 m^2, from the community based Walkerton Health Study (2002 to 2008) Follow-up: 7 yr	> 5% per year	Clark *et al*[74,119]
	Taiwan 7968 civil servants and teachers ⩾ 50 years old (mean age: 57 years old) Follow-up: 15 yr	> 20% per year	Reviewed by KDIGO CKD Work Group[18]: Cheng *et al*[29]
	Taiwan 167 patients in a hospital based study	> 25% per year	Chen *et al*[85]
Review	Chronic kidney disease Lancet	> 4 mL/min per 1.73 m^2 per year	Levey *et al*[3]
Guideline	KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease KDIGO CKD Work Group	> 5 mL/min per 1.73 m^2 per year	Inker *et al*[10] KDIGO CKD Work Group[18]

CHS: Cardiovascular Health Study; KDIGO: Kidney Disease: Improving Global Outcomes; eGFR: Estimated glomerular filtration rate; CKD: Chronic kidney disease.

els[65,69]. Factors associated with eGFR decline and progressive albuminuria might overlap. During a 10-year follow-up, an observational study of 1682 type 2 diabetics with baseline eGFR ⩾ 60 mL/min per 1.73 m^2 reported that obese patients had a significantly faster age-adjusted annual eGFR decline[70]. A positive association between glycated hemoglobin (HbA1c) and CKD has also been observed in type 2 diabetics, even in the absence of albuminuria and retinopathy[52]. An association between blood glucose, low-density lipoprotein abnormalities, and the progression of renal damage in diabetes has been reported[71]. HbA1c was found to be independently associated with rapid renal function decline in a group of type 2 diabetics without symptomatic cardiovascular disease[72].

Albuminuria and eGFR are not only biomarkers for the diagnosis and categorization of CKD[4], but are also well-known predictors of renal function decline, ESRD, and death in type 2 diabetics[8,73]. Proteinuria is associated with rapid decline in renal function[49-53], and a previous study suggests that dipstick proteinuria measurement could be used as a screening tool for rapid renal function decline[74].

Abnormalities in cardiovascular function

CKD shares many risk factors with cardiovascular disease[72,75], and dysfunction in one system can often lead to dysfunction in the other[49]. In patients with concomitant hypertension and type 2 diabetes, the risk of progression to ESRD is 7 fold that for age-matched control subjects[49,76]. Hypertension is a significant risk factor for insufficient renal function, cardiovascular events, and death in patients both with and without type 2 diabetes[49,61,77-79]. Previous studies show that systolic blood pressure (SBP) and pulse pressure are stronger predictors than diastolic blood pressure of renal outcomes, and are independent risk factors in the rapid decline of eGFR in type 2 diabet-

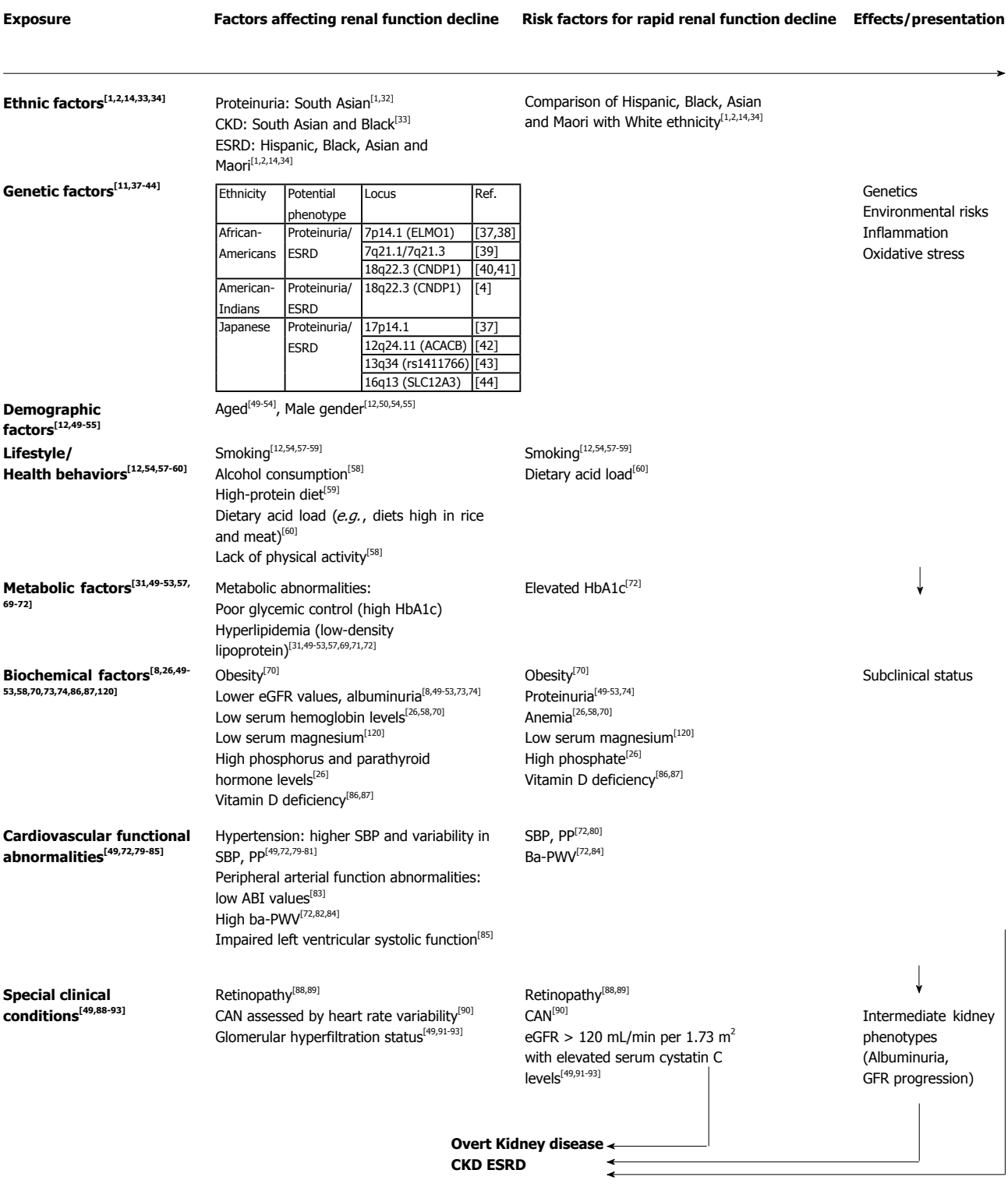

Figure 1 Conceptual model for diabetic kidney disease and potential risk factors of rapid renal function decline. CKD: Chronic kidney disease; HbA1c: Glycated hemoglobin; ESRD: End stage renal disease; eGFR: Estimated glomerular filtration rate; SBP: Systolic blood pressure; ba-PWV: Brachial-ankle pulse-wave velocity; PP: Pulse pressure; CAN: Cardiac autonomic neuropathy; ELMO1: Engulfment and cell motility 1; CNDP1: Carnosine dipeptidase 1; *ACACA*: *Acetyl-CoA carboxylase alpha*; *rs*: *RefSNP (Single Nucleotide Polymorphism) numbers*; SLC: Solute-carrier group.

ics[72,80], while another study suggests that both SBP and variability in SBP are risk factors in the development and progression of diabetic nephropathy[81].

In addition to blood pressure, peripheral arterial functional markers are also associated with renal function in type 2 diabetics[82]. A low ankle-brachial index was found to be significantly associated with a low eGFR[83]. Also, arterial stiffness is associated with incident albuminuria and decreased eGFR[72,84], and brachial-ankle pulse-wave velocity (ba-PWV) values are independently associated with rapid renal function decline in type 2 diabetics without symptomatic cardiovascular disease[72]. One study

reports that impaired left ventricular systolic function and increased ba-PWV are independently associated with a rapid decline in renal function[85].

Miscellaneous

Some other factors, such as low hemoglobin levels and electrolyte imbalance, may cause a rapid progression in diabetic kidney disease. Conditions including anemia, low serum magnesium levels, and high phosphorous and parathyroid hormone levels, are associated with rapid renal function decline in type 2 diabetics[26,58,70]. Furthermore, vitamin D deficiency associated with albuminuria was an independent risk factor in diabetic nephropathy after adjusting for demographic factors, hypertension, dyslipidemia, smoking status, and medication use[86,87].

Type 2 diabetic patients with additional microvascular complications, such as retinopathy or neuropathy, may also experience a rapid decline in renal function. Several studies have demonstrated that the rate of renal disease progression in type 2 diabetics with retinopathy is faster than that observed in those without retinopathy[88,89]; thus, screening for retinopathy may be helpful in identifying high-risk patients. Another study on cardiac autonomic neuropathy that assessed heart rate variability suggests that this is also an independent predictor of eGFR decline and could also be used as an identifying factor[90].

Special issues

Glomerular hyperfiltration and rapid renal function decline in type 2 diabetes: A longitudinal study of 600 type 2 diabetics with albuminuria < 200 μg/min, found that those with an eGFR > 120 mL/min per 1.73 m^2 had a higher risk of albuminuria progression (hazard ratio: 2.16) compared with those without baseline hyperfiltration; over a 4-year follow-up, renal function decline was relatively rapid, at an annual rate of up to 3.37 mL/min per 1.73 m^2[91]. Another study evaluated type 2 diabetic Pima Indians selected from participants in the Diabetic Renal Disease Study, with a baseline iothalamate clearance above the median for the entire study cohort (120 mL/min per 1.73 m^2) to give a study group with a normal or elevated GFR[92]. After a mean follow-up of 3.8 years, it was shown that directly measured GFR declined at 4.4% per year, and supposed that an increase in serum cystatin C provide means for detecting early renal function decline in diabetes[92]. Measurement of serum cystatin C may help to identify groups at high risk of renal function decline based on hyperfiltration status[49,93].

Non-albuminuric diabetic kidney disease: Renal insufficiency in the absence of albuminuria in patients with type 2 diabetes is another issue that should be noted. In a 1977 study of type 2 diabetic adults, 13% had an eGFR < 60 mL/min per 1.73 m^2, and 30% had neither albuminuria nor retinopathy[94]. Furthermore, data from UKPDS[12], DEMAND[13], and Atherosclerosis risk in Communities (ARIC)[52] studies suggests that the occurrence of renal impairment in type 2 diabetics without albuminuria is not unusual[49]. Microalbuminuria and reduced eGFR have been suggested as markers of different pathologic processes, with microalbuminuria associated with endothelial dysfunction and reduced eGFR being a renal manifestation of systemic atherosclerosis[49,95]. These patients are at higher risk of CKD progression, as the absence of proteinuria may lead to delays in the diagnosis and treatment of diabetic nephropathy[1,49].

POSSIBLE MANAGEMENT STRATEGIES

A number of therapeutic interventions for diabetic kidney disease have been developed over the past few decades[96]. Several studies have demonstrated increased activity in the renin-angiotensin-aldosterone system in diabetic patients with nephropathy[97,98]. Angiotensin-converting enzyme inhibitor (ACEI) and angiotensin receptor blocker (ARB) treatment for diabetics with hypertension can reduce renal damage and may reduce cardiovascular complications[97-99]; thus, ACEI or ARB are recommended as a first-line treatment for diabetics with hypertension[2,10,98,100,101]. However, based on the ONTARGET trial, acute dialysis, hyperkalemia, and hypotension tended to be more frequent with the use of both ACEI and ARB; thus, dual inhibition of the renin-angiotensin system is not recommended[102]. Primary multifactorial interventions aimed at slowing progression of diabetic nephropathy include combination therapy targeting hyperglycemia, hypertension, microalbuminuria, and dyslipidemia[59]. The Steno-2 study, of 151 type 2 diabetics with baseline microalbuminuria who underwent multifactorial treatment, reported that at a 7.8-year follow-up 46 patients showed remission to normoalbuminuria, improved hypertensive and glycaemic control were independent predictors for remission, and that kidney function may have been preserved through a slower rate of eGFR decline[103]. Other studies provide evidence that intensive multifactorial management is more effective than conventional treatment[104-107]. In addition to blood pressure, glycemic and lipid control, lifestyle modifications such as cessation of smoking, protein restriction in diets, weight reduction[2,59], light to moderate exercise[4], and vitamin C[104,105] and vitamin D supplementation[26], may be helpful in preventing or slowing the progression of diabetic kidney disease[2,26,59].

CONCLUSION

The progression of diabetic kidney disease is highly variable. According to the KDIGO 2012 clinical practice guidelines for the evaluation and management of CKD, a rapid decline in renal function was defined as a sustained decline in eGFR of > 5 mL/min per 1.73 m^2 per year. Associated risk factors in patients with type 2 diabetes include ethnic/genetic and demographic factors, lifestyle and health behaviors, advanced albuminuria, poor glycemic control, dyslipidemia, and some biochemical abnormalities. Diabetic patients with retinopathy or cardiac

autonomic neuropathy are at increased risk of a rapid decline in eGFR. Furthermore, those with glomerular hyperfiltration and elevated serum cystatin C may also be at increased risk of a rapid decline in renal function. Early detection of high-risk groups with a more aggressive multifactorial approach to renal and cardiovascular protection is important.

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REVIEW

Transdifferentiation of pancreatic α-cells into insulin-secreting cells: From experimental models to underlying mechanisms

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Abstract

Pancreatic insulin-secreting β-cells are essential regulators of glucose metabolism. New strategies are currently being investigated to create insulin-producing β cells to replace deficient β cells, including the differentiation of either stem or progenitor cells, and the newly uncovered transdifferentiation of mature non-β islet cell types. However, in order to correctly drive any cell to adopt a new β-cell fate, a better understanding of the *in vivo* mechanisms involved in the plasticity and biology of islet cells is urgently required. Here, we review the recent studies reporting the phenomenon of transdifferentiation of α cells into β cells by focusing on the major candidates and contexts revealed to be involved in adult β-cell regeneration through this process. The possible underlying mechanisms of transdifferentiation and the interactions between several key factors involved in the process are also addressed. We propose that it is of importance to further study the molecular and cellular mechanisms underlying α- to β-cell transdifferentiation, in order to make β-cell regeneration from α cells a relevant and realizable strategy for developing cell-replacement therapy.

Key words: α-cell; β-cell; Transdifferentiation; Diabetes mellitus; Cell-replacement therapy

Core tip: Recent works highlighted the phenomenon of transdifferentiation of pancreatic α cells into β cells, which has drawn much attention in the field. Considering that α-cell transdifferentiation could be used as a new strategy of cell replacement therapy for the treatment of diabetes, because of the presence of α cells in the pancreas of both type 1 and 2 diabetics, we believe that it is relevant to elucidate the cellular and molecular events in α- to β-cell conversion. Our review focuses on the recent experimental α-cell transdifferentiation models, highlighting the insight provided by these works into the candidates and contexts revealed to be involved in this process.

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INTRODUCTION

Pancreatic β cells are vital for glucose homeostasis. They are capable of producing and secreting insulin, a peptide hormone, in respond to high blood glucose levels. Insulin acts on diverse tissues to stimulate the metabolism of glucose[1,2]. Diabetes mellitus, becoming an epidemic in different parts of the world and a major public health challenge, is a carbohydrate metabolic disorder arising from failure of glucose homeostasis, with consequent hyperglycemia, resulting in severe complications affecting numerous tissues. The International Diabetes Federation estimated that 336 million individuals worldwide had diabetes in 2010. By 2030, this will have risen to 552 million[3]. The disease is characterized by either defective β-cell function as seen in Type 1 diabetes patients who have insufficient or even no β cells, or increased insulin resistance as observed in Type 2 diabetics who fail to maintain glycemic control because of, at least partially, insufficiency in β-cell mass or function. Consequently, there is an urgent need to search for efficient strategies to generate functional β-cells for cell replacement therapy.

The current strategies of generating new β cells can be outlined mainly in the following three ways[2,4]: (1) pluripotent stem cell differentiation: with the combined use of different factors, a pluripotent stem cell can be directed to differentiate into the cells with insulin-producing capability. Although such a directed differentiation seems to mimic normal pancreatic development, functional β cells can currently only be differentiated through a lengthy transplantation step; (2) inducing cell replication in existing β cells: this may be conducted either *in vitro* or *in vivo* using different agents or factors, but caution should be taken to avoid neoplastic transformation; and (3) reprogramming a differentiated cell by using genetic factors to induce a pluripotent state and factors driving a specific differentiation program. Reprogramming of acinar cells to generate β cells has proved to be successful *in vivo*[5]. More recently, a new strategy, the transdifferentiation of fully differentiated α cells into β cells, has emerged.

Transdifferentiation was originally defined as the change in a given adult cell from its initial differentiated state into another[2]. The most well known cell transdifferentiation phenomenon comes from the regenerative ability seen in urodele amphibians, which can regenerate their limbs, jaws, lens and large sections of their hearts. It is generally thought that transdifferentiating cells may go firstly through dedifferentiation, then proliferation and finally redifferentiation stages. Transdifferentiation can be distinguished from the above-mentioned directed stem cell differentiation by the fact that the initial cells are not "undifferentiated". Consequently, transdifferentiated cells are not systematically clonogenic. Although different examples of transdifferentiation were cited[2], it remains uncertain whether "natural" transdifferentiation can actually occur in mammals. More interestingly, recent studies have reported several experimental transdifferentiation models triggered either by drastically changing cellular and/or tissue contexts, or by directly altering molecular programs governing the cellular differentiation state (often referred to as cell conversion). Most notably, it is known that acino-ductal transdifferentiation can be seen in the case of severe tissue injury in the pancreas[6,7]. The treatment of rats with a copper-deficient diet resulted in the appearance of hepatocytes in the pancreas, whereas a reversed transdifferentiation was observed in the treatment of rats with polychlorinated biphenyls[8]. Experimental works have shown that either the pancreatic acinar tumor cell line AR42-J[9], or freshly isolated adult acinar cells[10] can transdifferentiate into hepatocytes *in vitro*. It was also reported that, under certain cell culture conditions, AR42-J cells were seen to display endocrine cell features[11,12]. Similarly, with the use of epidermal growth factor- and leukemia inhibitory factor-supplemented cell culture medium, it was reported that pancreatic exocrine cells were transdifferentiated into insulin-producing cells[13]. The phenomenon may also occur *in vivo*, the cells coexpressing transiently exocrine and endocrine markers being observed in rats that were subject to duct ligation[14-16], and in mice treated with alloxan[17]. Considering the particular role of Ngn3, its ectopic expression has been explored to trigger transdifferentiation of adult human duct cells into endocrine cells[18]. Finally, it is also speculated that β-cell mass increase seen in rats chronically infused with glucose may imply transdifferentiation as mechanisms of adaptation[19,20].

More interestingly, several laboratories have reported the phenomenon of transdifferentiation of pancreatic α cells into insulin-secreting cells (Table 1), which has been observed in different experimental settings[21-36]. Because of the close developmental and physiological relationship between these two cell lineages, and the presence of α cells in the pancreas of Type 1 and 2 diabetes patients, α-cell transdifferentiation draws much attention in the field of β-cell regeneration. Here, we review in detail these different models.

EXPERIMENTAL MODELS DISPLAYING β-CELL TRANSDIFFERENTIATION

Altered cross-regulatory circuit between Arx and Pax4

A number of studies have demonstrated that, during development, the influence of several transcription factors successively directs progenitor cells toward pancreatic, and ultimately islet endocrine cell fates. A complex network of transcription factors, including Arx and Pax4, progressively and differentially promotes particular endocrine fates[21,22]. In mice lacking Arx, β- and δ-cell

Table 1 List of some experimental models of α-cell transdifferentiation

Experimental model	Phenotype	Intermediate cells	α-cell proliferation	Ref.
Pax4 overexpression	Converts progenitor cells into α and subsequently β cells	Very few	-	[24]
Arx inactivation	α- to β-like conversion	+	-	[25]
Arx inactivation; Pdx1;Arx double mutant	α- to β-like conversion	+	-	[26]
Pdx1 overexpression	α- to normal β cell conversion	Numerous mantle-located Gcg + Ins + cells were detected in P1-P12	-	[28]
PDL + alloxan	A large number of new β cells arising from adult α cells within 14 d	58% of Ins+ cells coexpressed glucagon	-	[30]
Extreme β-cell loss	α- to β-cell transdifferentiation	+	-	[29]
Treatment with histone methyltransferase inhibitor	α- to β-cell conversion	Colocalization of both glucagon and insulin in human and mouse islets	-	[36]
Ablation of glucagon gene	Normoglycemia and hyperplasia of pancreatic α cells	+	+	[31]
Ablation of glucagon receptor ($Gcgr^{-/-}$)	Lower blood glucose, hyperglucagonemia, and pancreatic α-cell hyperplasia	Few scattered Gcg + Ins + cells or not mentioned	+	[27,32-34]
Impaired glucagon synthesis ($SPC2^{-/-}$)	Normoglycemia, hyperplasia of pancreatic α and δ cells	Not mentioned	+	[37]
Disturbed glucagon pathway [Liver-specific G(s)alpha deficiency]	Hypoglycaemia, hypoinsulinemia, pancreatic α-cell hyperplasia		+	[38]
Men1 inactivation	α-cell transdifferentiation, α-cell hyperplasia and development of glucagonoma and insulinoma	+	+	[39]

fates were found to be favored at the expense of α-cell genesis, while the total endocrine cell content remained normal[21]. Conversely, in the absence of Pax4, β-cell loss was observed accompanied by an increase in α-cell number[22], indicating an inhibitory, cross-regulatory circuit between Arx and Pax4[23].

Interestingly, Collombat *et al*[24] demonstrated that ectopically expressed Pax4 in endocrine precursor cells and α cells in the mouse resulted in the conversion of these cells into insulin-producing cells. As early as 1 wk postpartum, a 50% enlargement in islet size was outlined, with the islets containing increased numbers of insulin- and Pax4-positive cells compared with controls, and the number of glucagon-producing cells reduced by 77%. An age-dependent increase in islet size and the number of insulin-producing cells was observed. The latter exhibited most β-cell features, suggesting that, upon Pax4 ectopic expression, adult glucagon-expressing cells were continuously converted into cells exhibiting a β-cell phenotype. The lack of glucagon-producing cells resulted in an apparent adaptive neogenesis of α cells. The authors provided evidence suggesting that such a conversion triggered by Pax4 ectopic expression in α cells was sufficient to alleviate the diabetic condition resulting from massive β-cell destruction in the mouse.

More recently, Wilcox *et al*[25] showed that ablation of Arx in neonatal α-cells resulted in an α-to-β-like conversion through an intermediate bihormonal state, while short-term ablation of Arx in adult mice did not. However, Courtney *et al*[26] showed that selective *Arx* disruption in α cells at any age could elicit the conversion. It is important to note that such a conversion induced duct-lining precursor cells to differentiate to endocrine cells. The α cells thus generated were subsequently converted into β-like cells because of Arx inactivation. Using conditional *Arx* and *Pax4* double mutants, Courtney *et al*[26] provided evidence showing that Pax4 was dispensable for this regeneration process, suggesting that Arx could be the main trigger of α-cell conversion into β-like cells. Importantly, Arx disruption in α cells was able to reverse mouse diabetes resulting from β-cell depletion.

α to β cell reprogramming by forced PDX1 expression

Vuguin *et al*[27] performed ectopic Pdx1 expression from Ngn3-positive endocrine progenitors (*Neurog3*^{Cre}-*Pdx1*^{OE} mice). They detected a slight increase in β-cell number accompanied by a reduced α-cell number during the embryonic period[28]. At each stage, the combined number of α and β cells in *Neurog3*^{Cre}-*Pdx1*^{OE} mice was similar to that in controls, despite a significant difference in the α- to β-cell ratio, strongly suggesting a scenario of lineage diversion, where one cell population expands at the expense of the other under a constant total cell number. Two phases of lineage conversion were identified, contributing to a complete α-cell loss by the early adult stage. First, a significant decrease in glucagon-positive cell number (47% in the control reduced to 35% in mutant mice) and accompanying increase in insulin-positive cells was detected in the E16.5 *Neurog3*^{Cre}-*Pdx1*^{OE} pancreas, shortly after the peak of Neurog3 expression at approximately E15. Second, a major progressive loss of glucagon-positive cells in parallel with increased insulin-positive cell numbers was detected at P1-P12. Coexpression of insulin and α-cell-specific factors such as Arx, suggesting an early movement toward β-cell-directed transdifferentiation, was not detected at the first stage. Importantly, numerous mantle-located glucagon- and insulin-positive cells were detected in the second stage, representing intermediate

state α cells undergoing conversion, suggesting that the suppression of glucagon and the induction of insulin occurred concurrently. Intriguingly, when activating Pdx1 in the differentiated or mature glucagon-expressing α cell, the efficiency of the occurrence of α-to-β conversion was very much impaired, even absent. The work suggests that Pdx1 alone may play a strong role in regulating the cell differentiation program of islet-cells.

Near complete β-cell ablation

Thorel *et al*[29] have generated an elegant mouse model which allows nearly total β-cell ablation using the diphtheria toxin receptor system. The massive β-cell destruction thus obtained resulted in heterologous β-cell formation. Surprisingly, the majority of newly formed β cells originated from former glucagon-producing cells. By using cell lineage tracing, they demonstrated that, upon near total loss of β cells, genetically marked α cells rapidly began firstly to coexpress Nkx6.1, then coexpress insulin and the adult β-cell markers Pdx1, Nkx6.1 and Glut2, subsequently forming the majority of the regenerated β cells. Importantly, when α cells were ablated together with β cells, bihormonal cells expressing both glucagon and insulin were no longer observed. The work may also suggest that, in this particular experimental setting, a complete lack of local insulin signaling would elicit the interconversion between α- and β-cells. It would be interesting and challenging to use this model to further study the process and the mechanisms of α-cell transdifferentiation.

Pancreatic duct ligation + alloxan treatment

Chung *et al*[30] generated another pancreas and β-cell-deficient mouse model to study the origin and extent of adult β-cell regeneration. To this end, they used the β-cell specific toxin alloxan to ablate β cells, and, subsequently, carried out pancreatic duct ligation (PDL) to stimulate β-cell neogenesis. They reported that more than half (58%) of insulin-positive cells coexpressed glucagon one week after PDL and alloxan treatment. Moreover, they found that some glucagon-positive cells coexpressed β-cell-specific transcription factors, such as Pdx1 and Nkx6.1, suggesting a transitional stage during the conversion. Later, cells coexpressing insulin and glucagon were found. Interestingly, these insulin-positive cells expressed MafB, but afterward switched from MafB to MafA expression, suggesting that they were initially immature, and became mature over time. Unfortunately, cell lineage tracing was not performed in this model.

Glucagon pathway deficiency models

Mice with glucagon signaling deficiency, due to the inactivation of either the *Glucagon* gene[31] or its receptor (*GCGR*)[27,32-34], impaired glucagon synthesis[37], or a disturbed glucagon pathway[38], display common features. These include lower blood glucose levels, improved glucose tolerance with relatively normal insulin levels, and, in particular, α-cell hyperplasia and even tumorigenesis[33], accompanied by hyperglucagonemia and, in some of these models, scattered intermediate cells coexpressing insulin and glucagon. However, full transdifferentiation of α cells into β cells has never been demonstrated in the above models. Most probably, the fact that islets were often clustered near ductal tissue, and glucagon staining was seen along and budding from ductal epithelium or within exocrine tissue, suggests that the islet neogenesis could be the cause of increased α-cell mass.

Transdifferentiation from α cells to insulin-expressing cells triggered by Men1 disruption

In our previous study, we demonstrated the phenomenon of transdifferentiation in a mouse model where the *Men1* gene, a tumor suppressor in many types of endocrine cells, is specifically disrupted in pancreatic α cells[39]. Our analyses of pancreata from aging mutant mice showed that, in spite of the α-cell specificity of the *GluCre* transgene, both glucagonomas and insulinomas, as well as mixed islet tumors, were observed in mutant mice older than 6 mo of age. More interestingly, starting from as early as 2 mo of age well before tumor onset, cells sharing characteristics of both α and β cells, and coexpressing insulin and glucagon could be identified. Importantly, using a cell lineage tracing approach, we showed that these intermediate cells and insulinoma cells were both derived from *Men1*-deficient α cells. Furthermore, our data suggest that Pdx1, MafA and Ngn3 expression did not seem to be involved in the initiation of this transdifferentiation[39]. Intriguingly, although many *Men1*-deficient α cells transdifferentiated into insulin-secreting cells, some maintained their α-cell identity. This may indicate that *Men1*-disruption *per se* does not systematically lead to α-cell transdifferentiation, but rather affords the pathophysiological conditions to allow the transdifferentiation to occur. Other factors, independent of *Men1* disruption, may, therefore, play a crucial role in the initiation of the transdifferentiation. Using this model, where transdifferentiating cells are numerous before the development of tumors, to search for these factors would be of help in further deciphering the cellular and molecular basis of α-cell transdifferentiation. The identification of such factors would be crucial to determine the conditions favorable for α-cell transdifferentiation, while avoiding the known tumorigenic effect of *Men1* inactivation in islet cells.

CLUES TO OTHER FACTORS AND UNDERLYING MECHANISMS IMPORTANT FOR TRANSDIFFERENTIATION

The data from the above mouse models displaying experimental transdifferentiation of α cells into β cells suggest that α cells could possess intrinsic abilities to allow their conversion under certain circumstances, giving rise to an adaptive response to β-cell loss or deficiency. While these

models highlighted the genetic factors directly involved in such a process, they also provided clues as to other factors that may or may not participate in α-cell transdifferentiation.

Cell dedifferentiation

It is generally considered that natural transdifferentiation occurs in two steps: the dedifferentiation of the cell, followed by the differentiation of the dedifferentiated cell into the new lineage[40]. Although it is still unclear whether experimental transdifferentiation follows a similar course, the fact that no completely dedifferentiated α cells have been reported in the above experimental models seems to indicate that this may not be the case. Instead, it may be possible to directly convert one cell type into another. In this case, there could be a simultaneous switch from the inactivation of an old cell differentiation program into the activation of a new program. The existence of "intermediate cells" expressing both glucagon and insulin documented in several of these models even suggests that the initial activation of the new program may precede the complete inactivation of the old one. However, detailed cellular and molecular analyses are still required to allow a full understanding of the transdifferentiation procedure.

Epigenetic factors

Epigenetic mechanisms are known to play an important role in establishing and maintaining cell differentiation programs. Interestingly, a recent study demonstrated that α cells harbor bivalent chromatin signatures, containing both active and repressive histone markers, at genes that are active in β cells, such as Pdx1 and MafA[36]. The finding of α-cell plasticity may be supported by the fact that β-cell specific genes are likely ready to be activated. Moreover, they found that the repressed Pdx1 and insulin expression in α cells could be reactivated by treating islets with an inhibitor of histone methyltransferase. The work provides interesting clues into eventual cell reprogramming through epigenetic modifications[36].

Along with the above data, two other studies have demonstrated that changing histone methylation marks by deleting the *Dnmt1/3a* gene resulted in the transdifferentiation of β cells into α cells[41,42]. Indeed, detailed analyses showed that these two genes, together with other epigenetic factors, such as PRMT6, MeCP2 and HDAC1, play a crucial role in inhibiting the expression of transcriptional factors that may give rise to the activation of a cell differentiation program of other cell lineages, such as ARX in β cells. The loss of DNA methylation, therefore, results in the de-repression of these transcription factors, and the activation of the transcriptional program of other cell lineages. Thus, it would be interesting to investigate whether similar mechanisms could control α-cell identity.

Islet hormones and α-cell proliferation

Glucagon, insulin and GLP1: Glucagon was found to inhibit the formation of β cells converted from α cells upon Pax4 overexpression[24]. However, the phenomenon may be more directly related to the expansion of Ngn3 progenitors rather than the reprogramming itself, since virtually all α cells were converted to insulin-expressing cells by ectopic Pax4 expression, and mutant mice displayed hypoglucagonemia. Furthermore, in the *Men1* disruption-mediated transdifferentiation model, the very high levels of glucagon did not prevent α-cell transdifferentiation[39]. As for the potential inhibitory role of insulin deduced from the work by Thorel *et al*[29], the quasi absence of insulin does not seem to be a prerequisite for the occurrence of α-cell transdifferentiation, since the majority of experimental transdifferentiation models mentioned above display substantial levels of insulin. The existence of intra-islet GLP1 in many of the experimental transdifferentiation models makes it a plausible candidate involved in α-cell transdifferentiation. However, in aged GCGR knockout mice with extremely high levels of GLP1, only α-cell expansion, likely due to neogenesis, but not transdifferentiation was observed[34]. Altogether, the above data from different experimental transdifferentiation models indicate that islet hormones themselves, including glucagon, insulin and GLP1, may not be sufficient to be critically involved in the process.

α-cell proliferation: α-cell proliferation and hyperplasia, even neoplastic changes in some circumstances, were frequently found in various glucagon-deficient models. This raises the possibility that it may be required for, or even trigger, transdifferentiation. However, in the case of GCGR knockout mice, massive α-cell proliferation and neoplastic alteration did not lead to α-cell transdifferentiation. Importantly, a patient with a homozygote germline mutation of the GCGR gene displayed microglucagonoma and non-functional islet-tumor development, but no sign of α-cell transdifferentiation[43]. The data suggest that α-cell proliferation may be favorable for, but not systematically result in, the occurrence of transdifferentiation. At the same time, this highlights the potential deleterious effects of α-cell proliferation due to drastic glucagon pathway deficiency and/or massive α-cell loss.

Timing

In a recent work reported by Wilcox *et al*[25], the authors observed that embryonic α cells and adult α cells may react differently towards *Arx* disruption. Whereas the former were driven to convert to β cells, the latter seemed completely nonresponsive to the lack of ARX. However, similar work by Courtney *et al*[26] did not confirm this observation. The reason for the discrepancy remains unclear. Interestingly, another study, using ectopic Pdx1 expression in either pancreatic progenitors or in embryonic and mature α cells to reprogram the cells into β cells, also demonstrated that the efficiency of the reprogramming decreased when forced Pdx1 expression occurred later in embryonic development or in adult mice[28]. Col-

lectively, these studies highlighted the importance of timing in α-cell plasticity that should be taken into account for possible future clinical applications based on α-cell transdifferentiation.

CONCLUSION

Taken together, the above-mentioned recent studies highlighted the importance of both transcriptional factors and/or cofactors in maintaining cell differentiation status and in the physiological mechanisms involved in α-cell transdifferentiation. It would be vital and challenging for future studies to pinpoint the decisive factors from these two axes, and to provide insight into detailed mechanisms responsible for α-cell transdifferentiation. At the same time, past experience seems to indicate that some of the above-mentioned experimental conditions, such as PLD and glucagon pathway deficiency, may be more favorable for eliciting neogenesis, rather than α-cell transdifferentiation.

Because of their close ontogenic relation with β cells and unusual plasticity in responding to internal and external alterations, pancreatic α cells elicit much curiosity and clinical promise. In particular, the capacity for their transdifferentiation into insulin-secreting cells documented by several distinct models renders them a potentially relevant cellular basis for new strategies of β-cell regeneration. Deciphering detailed cellular and molecular mechanisms of the α-cell transdifferentiation process will be challenging for the field and crucial for future clinical applications.

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MINIREVIEWS

Place of sodium-glucose co-transporter type 2 inhibitors for treatment of type 2 diabetes

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Abstract

Inhibitors of sodium-glucose co-transporter type 2 (SGLT2), such as canagliflozin and dapagliflozin, are recently approved for treatment of type 2 diabetes. These agents lower blood glucose mainly by increasing urinary glucose excretion. Compared with placebo, SGLT2 inhibitors reduce hemoglobin A1c (HbA1c) levels by an average of 0.5%-0.8% when used as monotherapy or add-on therapy. Advantages of this drug class include modest weight loss of approximately 2 kg, low risk of hypoglycemia, and decrease blood pressure of approximately 4 mmHg systolic and 2 mmHg diastolic. These characteristics make these agents potential add-on therapy in patients with HbA1c levels close to 7%-8.0%, particularly if these patients are obese, hypertensive, and/or prone for hypoglycemia. Meanwhile, these drugs are limited by high frequency of genital mycotic infections. Less common adverse effects include urinary tract infections, hypotension, dizziness, and worsening renal function. SGLT2 inhibitors should be used with caution in the elderly because of increased adverse effects, and should not be used in chronic kidney disease due to decreased or lack of efficacy and nephrotoxicity. Overall, SGLT2 inhibitors are useful addition for treatment of select groups of patients with type 2 diabetes, but their efficacy and safety need to be established in long-term clinical trials.

Key words: Type 2 diabetes; Canagliflozin dapagliflozin; Weight loss; Hypoglycemia; Chronic kidney disease; Genital infection

Core tip: Sodium-glucose co-transporter type 2 inhibitors are recently approved drugs for type 2 diabetes with unique mechanism of action. In this minireview, the author provides a practical approach on how to select the best candidates for these drugs.

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INTRODUCTION

In healthy individuals, almost all glucose filtered by the kidneys is reabsorbed into the circulation, and less than 0.5 g of glucose per day is lost in urine[1]. Ninety per cent of glucose reabsorption from glomerular filtrate is mediated by sodium-glucose co-transporter type 2 (SGLT2) located in early segments (called S1 and S2) of proximal renal tubules[2,3]. The remaining 10% of filtered glucose is reabsorbed by means of SGLT1 located in late segment (S3) of proximal tubule[3]. SGLT2 inhibitors decrease hyperglycemia independently of insulin by lowering the renal threshold for glucose and therefore increasing urinary excretion of glucose[2]. Canagliflozin (Invokana) is the first SGLT2 inhibitor approved in the United States in March 2013 for treatment of type 2 diabetes[4]. Dapagliflozin

Table 1 Differences between canagliflozin and dapagliflozin

	Canagliflozin (Invokana)[4]	Dapagliflozin (Forxiga)[5]
Approved doses	Starting dose 100 mg tablet qd, taken before breakfast. If tolerated, dose can be increased to 300 mg tablet *qd*	Starting dose 5 mg tablet qd taken in the morning with or without food. If tolerated, dose can be increased to 10 mg tablet *qd*
Use in CKD	Contraindicated with eGFR < 45 mL/min per 1.73 m^2. Dose limited to 100 mg/d with eGFR of 45-59 mL/min per 1.73 m^2	Not recommended with eGFR < 60 mL/min per 1.73 m^2. No dose adjustment is needed with milder CKD
Hepatic impairment (Child-Pugh classification: A: mild, B: moderate, C: severe)	No dosage adjustment is needed with mild or moderate hepatic impairment. Not recommended with severe hepatic impairment	No dosage adjustment is needed with mild or moderate hepatic impairment. Start with smaller dose (5 mg/d) in severe hepatic impairment then the high-dose 10 mg/d if tolerated
Drug interactions	Use higher dose (300 mg/d) with UGT enzyme inducers (*e.g.*, rifampin) ↑ C max of digoxin by 36%. Use low starting digoxin doses, and monitor serum digoxin levels closely	No dose adjustment is needed when used with UGT enzyme inducers No interaction with digoxin
Effect on LDL-C levels (mean percentage change *vs* placebo)	↑ 4.5%-8%	↑ 3.9%
Possible increase in cardiovascular events	A trend toward increase in non fatal stroke and cardiovascular events (see text)	Not observed
Possible increase in cancer	Not observed	Possible increase in bladder cancer (0.17% *vs* 0.03% with placebo)

eGFR: Estimated glomerular filtration rate; Cmax: Maximum plasma concentration; CKD: Chronic kidney disease.

(Forxiga) was approved by the European Medicines Agency in November 2012, and by the Federal Drug Administration (FDA) in the United States in January 2014[5]. While head to head trials are lacking, some important differences exist between canagliflozin and dapagliflozin (Table 1). Many SGLT2 inhibitors such as empagliflozin, ipragliflozin, luseogliflozin are pending approval or still under development[2,6]. The main purpose of this review is to identify the optimum place of SGLT2 inhibitors in management of patients with type 2 diabetes based on both patients' characteristics and drug profile of SGLT2 inhibitors. More emphasis will be placed on the 2 approved SGLT2 inhibitors: canagliflozin and dapagliflozin.

SEARCH METHODOLOGY

PubMed search was conducted until July 2014 to identify all humans studies related to efficacy and safety of all SGLT2 inhibitors published in the English, Spanish and French literature. The search included all clinical trials of various SGLT2 inhibitors, pertinent guidelines of experts, review articles, prescribing information of canagliflozin and dapagliflozin are also reviewed. Search terms included "sodium glucose co-transporters", "diabetes mellitus", "canagliflozin", "dapagliflozin", "empagliflozin", "efficacy", "safety", "adverse effects", "cardiovascular effects", "mortality", "glycosuria".

Potential candidates for SGLT2 inhibitors

As add-on to other oral agents in patients with hemoglobin A1c levels of 7%-8.0%: In general, the efficacy of SGLT2 inhibitors is similar to metformin, sulfonylurea, pioglitazone, but canagliflozin may be slightly superior to sitagliptin [difference in hemoglobin A1c (HbA1c) 0.37%][7,8]. As result of their unique mechanism of action, SGLT2 inhibitors can be virtually combined with any other anti-diabetic therapy. A recent meta-analysis of 58 studies that included 8 different SGLT2 inhibitors showed that these agents reduced mean HbA1c levels by 0.79% when used as monotherapy and 0.61% when used as add-on treatment compared with placebo[7]. Because of universal agreement that metformin is the initial drug of choice for treatment of type 2 diabetes, the use of SGLT2 inhibitors as monotherapy is not justified except in selected patients who cannot tolerate metformin[9]. The place of SGLT2 inhibitors therefore is more appropriate as add-on therapy. For instance, after the addition of canagliflozin, dapagliflozin, and empagliflozin to patients with mean baseline HbA1c of approximately 8.0%, proportions of subjects who achieved HbA1c concentrations less than 7% were: 64% (*vs* 32% with placebo), 41% (*vs* 26% with placebo), and 32% (*vs* 9% with placebo), respectively[6,10,11]. In the previous 3 trials, background diabetes treatment consisted of metformin + pioglitazone, metformin alone, and metformin + sulfonylurea, respectively[6,10,11]. Clearly, in these studies, not all subjects achieved the HbA1c target of less than 7%. Hence, as baseline HbA1c levels become higher than 8.0% (*e.g.*, 8.5%-9%), the addition of a SGLT2 inhibitor may only improve, but unlikely optimize, glycemic control. In the latter setting, initiation of insulin is the most appropriate step.

Obese patients or patients concerned about weight gain: The use of SGLT2 inhibitors is consistently associated with mild weight loss of approximately 2 kg compared with placebo irrespective of presence or type of concomitant anti-diabetes therapy[7]. Weight loss becomes evident after 6 wk then usually reaches a plateau or slight-

ly rebounds after 26-32 wk until the end of follow-up at 104 wk[12]. The main cause of weight loss is increased urinary glucose loss, estimated to be approximately 100 g of glucose per 24 h[13]. Since each gram of glucose excreted in urine translates into a loss of 4 kcal, a loss of approximately 400 kcal/d is expected with SGLT 2 inhibitors[14]. Two studies using dual-energy X-ray absorptiometry show that approximately two-thirds of the reduction in body weight associated with administration of dapagliflozin and canagliflozin originates from fat mass, whereas the remaining one third is derived from lean body mass[15,16]. Another contributing factor to weight reduction may be fluid loss as result of the diuretic action of SGLT2 inhibitors, particularly during the initial rapid decline in body weight[15]. Since weight gain is a major unwanted effect of insulin therapy, addition of a SGLT2 inhibitor was evaluated in obese patients receiving high insulin doses (77 units/d)[12]. Thus, patients randomized to dapagliflozin lost an average weight of 1.4 kg without changing insulin requirements. Conversely, subjects randomized to placebo gained 1.8 kg, and their insulin requirements increased by 18 units/d[12]. Moreover, the HbA1c levels were 0.4% lower among dapagliflozin-treated group *vs* the placebo group[12]. Therefore, in insulin-treated patients concerned about weight gain, addition of a SGLT2 inhibitor may be a viable option.

Patients prone for hypoglycemia: The use of SGLT2 inhibitors is associated with low risk for hypoglycemia that is generally similar or slightly greater than placebo[11], similar to metformin[17], but 7-11 times less common than sulfonylurea (SU)[16,18]. Thus, in one trial, hypoglycemia occurred in 5% of patients randomized to canagliflozin 300 mg/d *vs* 34% of patients randomized to glimepiride (mean maximum dose 5.6 mg/d)[16]. SGLT2 inhibitors can be therefore a reasonable alternative to SU in patients with frequent hypoglycemia. The low hypoglycemic risk of SGLT2 inhibitors is attributed to the fact that these agents reduce renal glucose threshold to a range close to 76-90 mg/dL, *i.e.*, level that is above the plasma glucose concentration at which hypoglycemic symptoms occur[13,14]. Meanwhile, the incidence of hypoglycemia associated with SGLT2 inhibitors may increase in 3 conditions namely concomitant therapy with insulin and/or SU, in chronic kidney disease (CKD), and in the elderly. Thus, when dapagliflozin 10 mg/d was added to a background of insulin therapy, frequency of hypoglycemia was numerically greater among patients randomized to dapagliflozin than placebo, 57% and 52%, respectively[19]. With respect to CKD, in one study of patients with estimated glomerular filtration rate (eGFR) between 30 and 49 mL/min per 1.73 m^2, the proportions of subjects with documented hypoglycemia were higher with both doses of canagliflozin being 52% *vs* 36% with placebo[20]. Of note, the vast majority (96%) of the previous study population was also taking insulin or SU[20]. Finally, regarding advanced age, in a study of older patients (mean age 64 years), the incidence of hypoglycemia was 36% and 28% with canagliflozin 300 mg/d, and placebo, respectively[21].

Patients with uncontrolled hypertension: In one meta-analysis of 27 randomized trials, the use of various SGLT2 inhibitors was associated with mean reduction of systolic and diastolic blood pressure of 4.0 mmHg and 1.6 mmHg, respectively compared with baseline[22]. Only canagliflozin showed dose-response relationship with systolic blood pressure[22]. The decrease in blood pressure is most likely due to osmotic diuresis, but mild weight loss may be another contributing factor[13]. It is reassuring that the decrease in blood pressure was not associated by an increase in heart rate[8,23].

Patients in whom SGLT2 inhibitors may be used with caution

Women with history of mycotic genital infections and uncircumcised men: Increased vaginal fungal infection is the most common adverse effect of SGLT2 inhibitors reported by 11%-14% of patients who received canagliflozin or dapagliflozin compared with 2%-4% in subjects randomized to placebo or a comparator agent such as glimepiride or sitagliptin[8,16]. The increased genetic mycotic infection is most likely related to the increase in urinary glucose excretion induced by SGLT2 inhibitors. The median time of diagnosis was 19 d after the initiation of canagliflozin, and the most frequently isolated Candida species were Candida albicans (51%) and Candida glabrata (37%)[24]. Infection is frequently recurrent, and patients with previous history of genital mycotic infections are more prone to develop this type of infection[4,19,25].

Increased frequency of genetic mycotic infections also occurs in men exposed to SGLT2 inhibitors, albeit to a lesser extent than in women[25]. These include balanitis or balanoposthitis. In the trial of Cefalu *et al*[16], frequency of genetic mycotic infections in men exposed to canagliflozin 100 mg/d, canagliflozin 300 mg/d, and glimepiride was 7%, 8%, and 1%, respectively. Rates of infection are relatively higher in uncircumcised men and those with history of balanitis[4,25]. In general, genital mycotic events in both genders were considered mild to moderate in severity, were treated with topical or oral anti-fungal agents without interruption of the drug, and uncommonly led to withdrawals[8]. The frequency of UTI is also increased with the use of SGLT2 inhibitors, being 7.2%, 5.1%, and 4.2% among patients randomized to canagliflozin 100 mg/d, 300 mg/d, and placebo, respectively[23]. Candida spp. was cultured from the urine specimens of 4.4% of canagliflozin-treated patients compared with 1.1% of control subjects[26]. This increased frequency of candiduria may reflect contamination from vaginal colonization[26].

Elderly patients: Two main reasons make the use of SGLT2 inhibitors in the elderly not an attractive option: diminished efficacy and increased frequency of some adverse effects. Thus, mean reduction of HbA1c with the highest dose of canagliflozin (300 mg/d) *vs* placebo was

0.8% and 0.5% after 26 wk among patients younger than 65 years and those who were older than 65 years, respectively[21]. This decreased efficacy was also demonstrated in a pooled analysis of 4 other canagliflozin studies[27]. Likewise, in one trial of dapagliflozin, reduction in HbA1c levels in patients younger than 65 (mean age 58 years) and older than 65 (mean age 70 years) was 0.4% and 0.3%, respectively after 24 wk compared with baseline[28]. Since the anti-hyperglycemic action of SGLT2 inhibitors rely on enhancing urinary glucose excretion, the decreased efficacy of the agents with old age is in large part attributed to the reduction in eGFR that normally occurs with aging[21]. Besides decreased efficacy, available data suggest that several adverse effects of SGLT2 inhibitors may increase with advanced age. First, elderly patients exposed to SGLT-2 inhibitors are more prone for worsening renal function than younger patients. Thus, in patients aged 65 and older, renal impairment and renal failure occurred among 14.8% of patients randomized to dapagliflozin *vs* 8.0% with placebo, whereas corresponding proportions in patients younger than 65 were 4.7% and 0.4%[28]. Second, elderly patients receiving canagliflozin and dapagliflozin may be more prone for volume-depletion adverse effects such as hypotension, dizziness, and syncopy[4,5,27]. Third, as mentioned earlier, elderly patients may be more susceptible to hypoglycemia associated with SGLT2 inhibitors[21].

Patients with significant history of vascular disease: The Canagliflozin Cardiovascular Assessment Study (CANVAS) is an ongoing large randomized trial that primarily examines the effects of canagliflozin on cardiovascular events and mortality in patients with long-standing type 2 diabetes and elevated cardiovascular risk[29]. An imbalance in the incidence of cardiovascular events was recorded during the first 30 d of CANVAS. Thus, 13 of 2889 patients had an event in the canagliflozin group compared with 1 of 1441 patients in the placebo group yielding a hazard ratio of 6.5 (95%CI: 0.85-49.6). This imbalance was not evident after 30 d[7]. In addition, the FDA reported a trend toward an increase in nonfatal stroke in patients who received canagliflozin [HR = 1.46 (95%CI: 0.83-2.58)][7]. Regarding dapagliflozin, the limited available data is somewhat reassuring. Thus, one trial of older patients (mean age 64 years) with advanced type 2 diabetes and history of cardiovascular disease did not show difference in cardiovascular events or mortality between patients randomized to dapagliflozin compared to placebo after 52 wk of intervention[28].

Patients with osteoporosis: Incidence rate of bone fractures derived from pooled data of 8 trials were 18.7, 17.6, and 14.2 per 1000 patient years of exposure to canagliflozin 100 mg/d, 300 mg/d, and comparator, respectively[4]. In one study of patients with moderate renal impairment (mean age 67 years), 13 of 85 (7.7%) patients randomized to dapagliflozin experienced fracture compared to none of the 84 subjects randomized to placebo[30]. The reasons of excess fractures in patients exposed to canagliflozin and dapagliflozin are unclear. No notable changes in serum or urine calcium, 1.25 dihydroxy vitamin D, or parathyroid hormone were reported[19]. However, in 2 canagliflozin studies, there was a modest increase in one marker of bone resorption, serum collagen type 1 β-carboxy-terminal telopeptide[14,21]. Nevertheless, until further data become available, SGLT2 inhibitors should be used with caution in patients having history of osteoporosis or fractures.

Patients in whom SGLT2 inhibitors should be avoided

Patients with chronic kidney disease: As mentioned earlier, the risk of hypoglycemia associated with the use of SGLT2 inhibitors in patients with CKD is increased[20]. Other reasons to avoid the use of these drugs in CKD are decreased or lack of efficacy and worsening renal function. Thus, in patients with stage 3 CKD, defined as eGFR between 30 and 49 mL/min per 1.73 m^2, the efficacy of canagliflozin was only modest with mean HbA1c reduction of 0.4% as compared with placebo[20]. Furthermore, in another trial of patients with eGFR of 30 to 59 mL/min per 1.73 m^2, dapagliflozin did not have any significant effect on HbA1c levels compared with placebo[30]. This decreased or absent efficacy of SGLT2 inhibitors in CKD is most likely the result of reduction of renal glucose clearance as eGFR declines[21,31]. Patients with CKD are particularly susceptible to the nephrotoxic effects of SGLT2 inhibitors. Indeed, increase in serum creatinine, and decrease in eGFR were demonstrated after 1-3 wk of exposure to dapagliflozin and canagliflozin, respectively[20,30]. Therefore, the use of dapagliflozin and canagliflozin is contraindicated in patients with eGFR < 60 mL/min per 1.73 m^2, and 45 mL/min per 1.73 m^2, respectively[4,5].

Patients with high low density lipoprotein-cholesterol (LDL-C) concentrations: For unclear reason, canagliflozin was found to increase plasma levels of LDL-C in a dose-related fashion. In pooled data from 4 placebo-controlled trials, mean percentage increases over baseline values were 4.5% and 8% with 100 mg/d and 300 mg/d, respectively relative to placebo[4]. In one study of 26 wk-duration, slight increases in plasma levels of apolipoprotein B of 1.2% and 3.5% were reported among patients randomized to canagliflozin 100 mg/d, and 300 mg/d, respectively compared with 0.9% increase with placebo[23]. Canagliflozin also increased levels of high density lipoprotein-cholesterol, with mean percentage increase of 6.1%-6.8% relative to placebo[23], and 8%-9% relative to glimepiride[16]. Clearly, the increase in plasma levels of LDL-C and apolipoprotein B is concerning, and its impact on cardiovascular events needs to be carefully examined. The effect of dapagliflozin on LDL-C levels is inconsistent. In pooled data from 13 placebo-controlled trials, mean percentage increase in LDL-C levels was 2.9% in dapagliflozin groups *vs* -1% in placebo groups after 24 wk[5]. Yet, in one trial lasting 2 years, no change in LDL-C

levels was recorded in dapagliflozin-treated subjects[12].

Patients with history of bladder cancer: Possible increased risk of bladder cancer was observed in dapagliflozin trials[5]. Accordingly, dapagliflozin should not be used in patients with history of bladder cancer until further data become available[5].

OTHER LIMITATIONS OF SGLT2 INHIBITORS

Although almost all clinical trials of SGLT2 inhibitors are randomized and double-blind, they are sponsored by corresponding manufacturers, and therefore open to various bias, *e.g.*, using comparator drug in submaximal doses, or not mentioning its actual doses[16,18]. Moreover, the meta-analysis of Vasilakou *et al*[7] revealed that reduction in HbA1c levels by these agents may be overstated because of high discontinuation rates and handling missing data by the use of "last observation carried forward". Indeed, the latter method is considered inappropriate and can potentially inflate drug efficacy[32]. The high cost, and absence of long-term data (*e.g.*, 5 years or more) are further limitations of this new class of drugs.

CONCLUSION

Owing to their unique mechanism of action and acceptable efficacy, SGLT2 inhibitors represent a useful add-on therapy in patients with uncontrolled type 2 diabetes. Patient subgroups that would potentially benefit the most from this class are those with HbA1c levels in the range of 7%-8%, subjects concerned about weight gain, patients prone for hypoglycemia, or those with uncontrolled hypertension. On the other hand, these agents are not recommended in CKD, and should be used with caution in the elderly. It may be wise not to use canagliflozin in patients with established cardiovascular disease and high LDL-C levels until further data become available. The results of the ongoing large randomized trials should clarify the long-term safety of different members of SGLT2 inhibitors with respect to cardiovascular morbidity and mortality, incidence of cancer and fractures[29,33].

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Molecular mechanisms of AGE/RAGE-mediated fibrosis in the diabetic heart

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Abstract

Chronic hyperglycemia is one of the main characteristics of diabetes. Persistent exposure to elevated glucose levels has been recognized as one of the major causal factors of diabetic complications. In pathologies, like type 2 diabetes mellitus (T2DM), mechanical and biochemical stimuli activate profibrotic signaling cascades resulting in myocardial fibrosis and subsequent impaired cardiac performance due to ventricular stiffness. High levels of glucose nonenzymatically react with long-lived proteins, such as collagen, to form advanced glycation end products (AGEs). AGE-modified collagen increase matrix stiffness making it resistant to hydrolytic turnover, resulting in an accumulation of extracellular matrix (ECM) proteins. AGEs account for many of the diabetic cardiovascular complications through their engagement of the receptor for AGE (RAGE). AGE/RAGE activation stimulates the secretion of numerous profibrotic growth factors, promotes increased collagen deposition leading to tissue fibrosis, as well as increased RAGE expression. To date, the AGE/RAGE cascade is not fully understood. In this review, we will discuss one of the major fibrotic signaling pathways, the AGE/RAGE signaling cascade, as well as propose an alternate pathway *via* Rap1a that may offer insight into cardiovascular ECM remodeling in T2DM. In a series of studies, we demonstrate a role for Rap1a in the regulation of fibrosis and myofibroblast differentiation in isolated diabetic and non-diabetic fibroblasts. While these studies are still in a preliminary stage, inhibiting Rap1a protein expression appears to down-regulate the molecular switch used to activate the ζ isotype of protein kinase C thereby promote AGE/RAGE-mediated fibrosis.

Key words: Type 2 diabetes mellitus; Cardiac fibrosis; Fibroblasts; Advanced glycation end product; Rap1a; Extracellular matrix

Core tip: Chronic hyperglycemia is a characteristic of diabetes and one of the major causal factors of diabetic complications. In type 2 diabetes mellitus, mechanical and biochemical stimuli activated profibrotic signaling cascades resulting in myocardial fibrosis, impaired cardiac performance, and ventricular stiffness. Glucose nonenzymatically reacts with extracellular matrix (ECM) proteins forming advanced glycation end products (AGEs). AGE-modified collagen increases matrix accumulation and stiffness by engaging the receptor for AGE (RAGE), the receptor for AGE. To date, our understanding of the AGE/RAGE cascade remains imprecise. This review discusses the AGE/RAGE signaling cascade and proposes an alternate role for Rap1a in diabetic cardiovascular ECM remodeling.

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INTRODUCTION

Chronic hyperglycemia is one of the main characteristics of diabetes mellitus. There are two forms of the disease, which are classified based upon insulin dependence: type 1 diabetes mellitus (T1DM) or T2DM. T1DM is considered a progressive autoimmune disorder of the pancreas causing the destruction of islet β-cells and resulting in diminished insulin production. The subsequent insulin deficiency results in elevated blood glucose levels. T2DM is generally coupled with metabolic syndrome, which includes increased insulin resistance, hyperglycemia, obesity, dyslipidemia and hypertension. Persistent exposure to elevated glucose levels has been recognized as one of the major causal factors of diabetic complications resulting in pathologies, such as atherogenesis, myocardial infraction, stroke and diabetic cardiomyopathy[1]. In this review, we will discuss one of the major fibrotic signaling pathways, the advanced glycation end product (AGE)/the receptor for AGE (RAGE) signaling cascade driven by chronic hyperglycemia in T2DM, as well as propose an alternate pathway that may offer insight into cardiovascular extracellular matrix (ECM) remodeling.

FIBROBLAST MEDIATED ECM REMODELING

In the heart 70%-80% of the cellular mass is composed of myocytes, and the remaining 20%-30% the total cell number includes fibroblasts, vascular smooth muscle cells, and endothelial cells[2,3]. Fibroblasts are the most abundant cardiac cell types of the latter group, and these cells are accountable for homeostatic upkeep and pathological ECM alterations observed in the heart[2,3]. Fibroblasts also function as sensory cells recognizing mechanical and chemical changes within the cell's microenvironment[4]. Fibroblasts communicate with the surrounding ECM to maintain the structural arrangements of the heart as well as sustain vital cellular tasks, such as viability, proliferation, and motility[5].

In pathologies, like T2DM, where biochemical and mechanical stimuli alter the communication between the ECM and fibroblasts, profibrotic signaling cascades are subsequently activated to elevate fibrotic accumulation and subsequently increased heart stiffness[4,6,7]. Increased ECM deposition and accumulation may result from either enhanced matrix protein synthesis and/or decreased structural degradation. With elevated matrix production and accumulation structural ECM rearrangements would cause alterations in fibroblast-matrix interactions. These changes often result in transformations in fibroblast phenotype. Fibroblast isolates from hypertensive animals as well as from infarcted regions of the heart exhibit increased matrix production and accumulation, reduced cell migration, and greater contractility[8-10]. In these instances, changes in fibroblast phenotype correspond to increases in fibroblast to myofibroblast differentiation. Myofibroblasts are defined as a "stressed" fibroblast having increased matrix production as well as enhanced contractile properties[11-13].

This cell type is not commonly found in healthy myocardium, however upon pathological cardiac injury, myofibroblast populations will increase in the myocardium from differentiated interstitial and adventitial fibroblasts[13]. While initially beneficial in pathologies requiring enhanced scar formation to maintain organ integrity (*e.g.,* myocardial infarction), myofibroblasts become detrimental to organ function if an increased population of myofibroblasts persists. Due to the high glucose levels seen in diabetic patients, studies have demonstrated an elevated synthesis and accumulation of the ECM, otherwise known as fibrosis, to increase ventricular stiffness to negatively impact heart function[14,15]. Ultimately, myofibroblasts are detrimental due to their critical role in cardiac pathology and remodeling, and in certain environments, such as diabetes mellitus, improper regulation of myofibroblasts leads to maladaptive tissue remodeling[13,16].

HYPERGLYCEMIA AND AGE

Numerous reports have documented chronic hyperglycemia is the causative agent responsible nonenzymatic formation of AGEs on substrates resistant to turnover, such as collagen[13]. These modifications will not only reinforce the ECM by adding surplus collagen structural crosslinks but also as a RAGE agonist. Chronic hyperglycemia, as observed in T2DM patients, increases the generation of AGEs. High levels of glucose nonenzymatically react with long-lived proteins forming reversible Schiff base intermediates and eventually, Amadori compounds[17]. Amadori products will undergo additional chemical alterations to be converted to nonreversible crosslinked AGES[17]. AGEs are also found to accumulate in normoglycemic patients as a result of longevity. Under high glucose settings observed in diabetics, AGE formation is accelerated, resulting in cardiac dysfunction as well as interstitial fibrosis[17-20]. AGE-modified collagen causes an increase in matrix stiffness causing it be resistance to hydrolytic turnover, resulting in an accumulation of ECM[17,21].

In vivo and *in vitro* studies demonstrate that AGEs account for many of the diabetic cardiovascular complications through their engagement of RAGE[22]. RAGE is capable of binding to multiple ligands. Under normoglycemic conditions the receptor is ordinarily expressed at reduced basal levels, however due to aging and to chronic hyperglycemia, RAGE expression is increased[17,20]. AGE/RAGE cascade activation promotes fibrosis growth factor secretion, increased matrix deposition progressing to multi-organ fibrosis, as well as increased RAGE expression[21,23-25]. Increased AGE crosslinks, AGE/RAGE cascade activation, and increased matrix accumulation have been correlated with the development of cardiovascular complications by increasing diastolic left ventricular stiffness[21,25,26]. AGEs have been demonstrated to increase expression of multiple collagen types, decrease proteo-

glycans synthesis, as well as generate ECM crosslinking. Interestingly, AGEs can be bound to other macromolecules to compound their negative impacts on a number tissues[15,27,28]. Also, they have been shown to perturb cell-matrix interactions, alter cell adhesion, and vascular permeability. Many of the maladaptive ECM alterations have been shown to be relatively corrected by disrupting the AGE/RAGE signaling cascade[29]. Therefore, the AGE/RAGE cascade provides a hypothetical focus for the management of diabetes-mediated ECM related cardiovascular diseases.

AGE/RAGE SIGNALING PATHWAY

Increased AGE/RAGE signaling has been demonstrated to promote key pathways that upregulate ECM protein expression and accumulation. In addition, activation of downstream signaling kinases such as p38, extracellular signal-regulated kinase 1/2 (ERK 1/2), nuclear factor-kappaB (NF-κB), and c-Jun N-terminal kinase (JNK), have been shown to mobilize multiple transcription factors to stimulate expression of growth factors and ECM protein accumulation[30-33]. Numerous studies have suggested that AGE/RAGE signaling pathways are ligand- and cell type dependent. For example, in endothelial progenitor cells, AGE/RAGE cascade activation inhibited migration while promoting apoptosis to further atherosclerosis in diabetic patients[34,35]. Upon treatment with anti-RAGE peptide antibodies, AGE/RAGE signaling pathway was down regulated and diabetic atherosclerotic lesions and vascular injury was significantly attenuated[34]. It also has been reported that AGE/RAGE is implicated in diabetic related macrovascular complications, arterial injury, as well as the progression of diabetic nephropathy and retinopathy[36]. In a T2DM leptin receptor deficient (*db*/*db*) mouse model, using RAGE blocking antibody, left ventricular diastolic chamber stiffness and the cardiac systolic function was attenuated in conjunction with reduced fibrosis. It has been proposed the multiple outcomes of AGE/RAGE signaling operate through protein kinase C (PKC). Utilizing cell culture experiments to model T1DM and T2DM hyperglycemic growth conditions *in vitro*, PKC activity was increased and followed by subsequent activation of various prostaglandins, cytokines, and increased ECM protein expression[22]. Immunoblotting experiments using of cellular lysates revealed PKC-α, -β Ⅰ, -β Ⅱ, -δ, -ε, and -ζ isoform activity was increased in endothelial cells[37].

The PKC kinase family is defined based upon their second messenger requirements. The conventional PKC family, which includes PKC-α, -β Ⅰ, -β Ⅱ, and -γ, is stimulated by calcium, phosphatidylserine, diacylglycerol, or phorbol-12-myristate-13-acetate. Members of the novel PKC group, which includes -δ, -ε, -θ and -η are also activated by the above ligands with the exception of calcium. The atypical PKC family, which includes -ζ and -ι/λ, cannot be activated by any of the above second messengers[38]. To date, PKC isoform activation has been associated with vascular alterations, including increased permeability, contractility, ECM synthesis, cell growth, and apoptosis[37], and these perturbations in vascular cell homeostasis have been shown to be mediated by differing PKC isoforms[37]. Of these isoforms, PKC-β and PKC-ζ emerged as a preferred substrate in the aortic and cardiac tissue of diabetic mice[39,40]. Additional examination of multiple PKC isoforms has identified of PKC-ζ as the most plausible target for RAGE phosphorylation[41].

PKC-ζ is involved in propagating a multiple of cascade pathways that lead to mitogen-activated protein kinase (MAPK) activation. The MAPK family plays a pivotal role in numerous cellular processes, including development, phenotype differentiation, and ECM protein synthesis. In a study by Koya *et al*[37], ERKs were demonstrated to be activated in a PKC-dependent manner. ERKs are a subfamily of MAPKs involved in signaling cascades responsible for multiple cellular functions, such as differentiation and proliferation. Stimulation of ERK signaling cascades involve activation of a molecular switch, Raf, to trigger a stepwise serine kinase cascade through activation of Raf, MAPK kinase kinase, MAPK kinase, MAPK, and ERK[42]. Activated ERK will translocate into the nucleus to activate transcription factors to initiate cellular proliferation, differentiation, and matrix accumulation[43-45].

AGE/RAGE and PKC-ζ signaling cascades have been demonstrated to increase ERK activation, both independently as well as synergistically; thereby PKC-ζ serves as a common molecular mediator between these two different cascades[46,47]. Phosphorylation of RAGE at Ser391 is a ligand-dependent mechanism that is required to perpetuate AGE/RAGE signaling[41]. PKC-ζ has been demonstrated to phosphorylate Ser391 of the intracellular RAGE domain. However in order for this to occur, PKC-ζ must be activated by Ras, a small GTPase, to initiate the cascade[41]. Recently, our lab and others have found that Rap1a, a small Ras-like GTPase, may also play a role in AGE/RAGE signaling in diabetes.

RAP1A: A MOLECULAR SWITCH

Rap1a, member of the Ras superfamily, operates as a binary molecular switch. This relay system is capable of transmitting a number of diverse signals from members of the Ras superfamily to effect changes in nuclear transcription, thus coupling extracellular stimulation to intracellular signaling cascades. In fact, Rap1a has been demonstrated to participate in hypertrophic pathways, integrin-mediated adhesion, cell attachment, migration, and cell junction formation. Studies have shown that Rap1a induced-ERK1/2 activation contributes to vascular pathologies as well as plays a role in the cardiovascular ion channels responsible for rhythmic heart function[48].

Rap1a utilizes a guanine nucleotide exchange factors (GEFs), that causes the dissociation of a bound GDP allowing for a new GTP molecule to bind. GTPase-activating proteins (GAPs) will then hydrolyze the newly

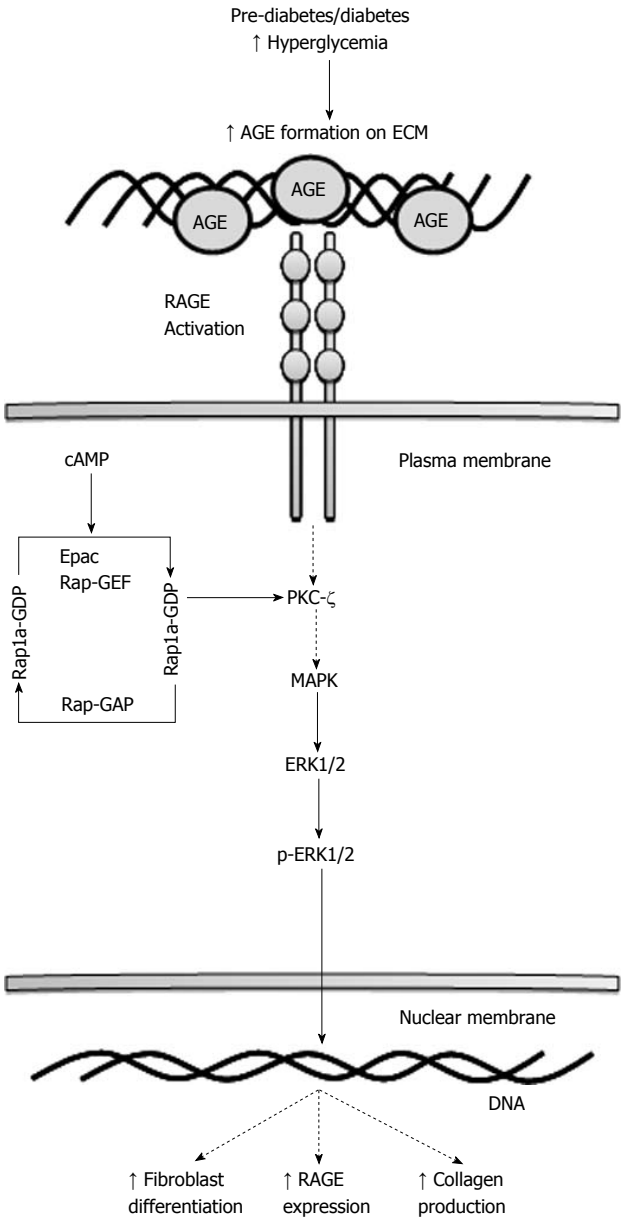

Figure 1 Rap1a in advanced glycation end product/the receptor for advanced glycation end product signaling. A potential role for Rap1a as a molecular switch mediating the AGE/RAGE signaling pathway in type 2 diabetes mellitus. Increased Rap1a activity may stimulate PKC-ζ to further promote matrix accumulation, RAGE expression and fibroblast differentiation to myofibroblasts. AGE: Advanced glycation end product; RAGE: The receptor for AGE; ECM: Extracellular matrix; cAMP: Cyclic AMP; GAP: GTPase-activating protein; PKC-ζ: The ζ isotype of protein kinase C; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase.

bound GTP to GDP forcing the cycle to run in one direction. In this capacity, Rap1a rotates between the inactive GDP-bound and the active GTP-bound substrate. In addition, Rap1a has been demonstrated to be activated by at three second messengers, specifically cyclic AMP (cAMP), calcium, and diacylglycerol[49]. It is now recognized that a number of GEFs can be directly activated by cAMP whereby cAMP binding causes a conformational change in the GEF permitting nucleotide exchange. Of particular interest are the GEFs known to activate Rap1a. These are commonly referred to as cAMP-GEF or more specifically Epac (Exchange Protein directly Activated by cAMP). Epac proteins have been demonstrated to bind cAMP and activate Rap1a GTPases[50]. Conversely, Rap1a-GAP will hydrolyze GTP at the asparagine side chain, thereby rendering Rap1a inactive.

The dynamic control of Rap1a activation has been shown to be facilitated by protein kinase A (PKA) and Epac through cAMP-dependent cascades[51]. Both PKA and Epac proteins contain a cAMP binding domain and are sensitive to fluctuations to mediate Rap1a activation[48]. While PKA can phosphorylate the C-terminus of Rap1a, PKA-mediated activation is not necessary for cAMP stimulation of Rap1 by Epac. In fact, there have been extensive studies that have established Epac's involvement in various cAMP-related cellular functions, such as cellular adhesion, that were previously attributed to PKA[52,53]. These cAMP sensitive proteins may act independently, synergistically, or possible antagonistically depending upon cellular distribution, concentration, and location to regulate Rap1a-mediated cellular functions. Our understanding of the Rap1a pathway is centered on the biological responses elicited by PKA-dependent pathways triggering downstream ERK1/2 activation[30]. However, recent studies have suggested a PKA-independent pathway for Epac-Rap1a activation of downstream signaling effectors[54]. Precise investigation of the discrete role and involvement of Rap1a is necessary within a number of signaling model systems.

AGE/RAGE and Rap1a-induced ECM accumulation in diabetes

To date, there is paucity in the literature describing the interactions between Rap1a and the AGE/RAGE signal pathway in T2DM. Early studies described Rap as being up-regulated in multiple organs of diabetic rats[55]. Of note, these studies also demonstrated that diacylglycerol can activate a Rap/Raf/MAPK-mediated signal cascade through PKC, however no specific PKC isoform was identified[55]. Furthermore, in a study by Panchatcharam *et al*[56], increased Rap1 expression was reported in smooth muscle cells under hyperglycemic conditions, yet no distinction between Rap1a or Rap1b subtypes was made. Taken together, there is evidence that Rap1a under hyperglycemic conditions will increase downstream kinase activity *via* ERK1/2 activation, and these events would ultimately influence other signaling pathways, including the AGE/RAGE cascade, to promote ECM accumulation to contribute to cardiac complications in diabetic patients.

Both the AGE/RAGE signaling cascade and Rap1a utilize and activate similar signaling pathways, such as ERK1/2 MAPK, NF-κB and JNK, which are involved in cell growth, ECM synthesis and myofibroblasts differentiation. It has been demonstrated that fibroblasts treated with transforming growth factor-β, a known fibrosis mediator, myofibroblasts differentiation and ECM deposition is increased[17,57]. Furthermore, studies by Yan *et al*[57], showed that major molecular mediators, like ERK1/2

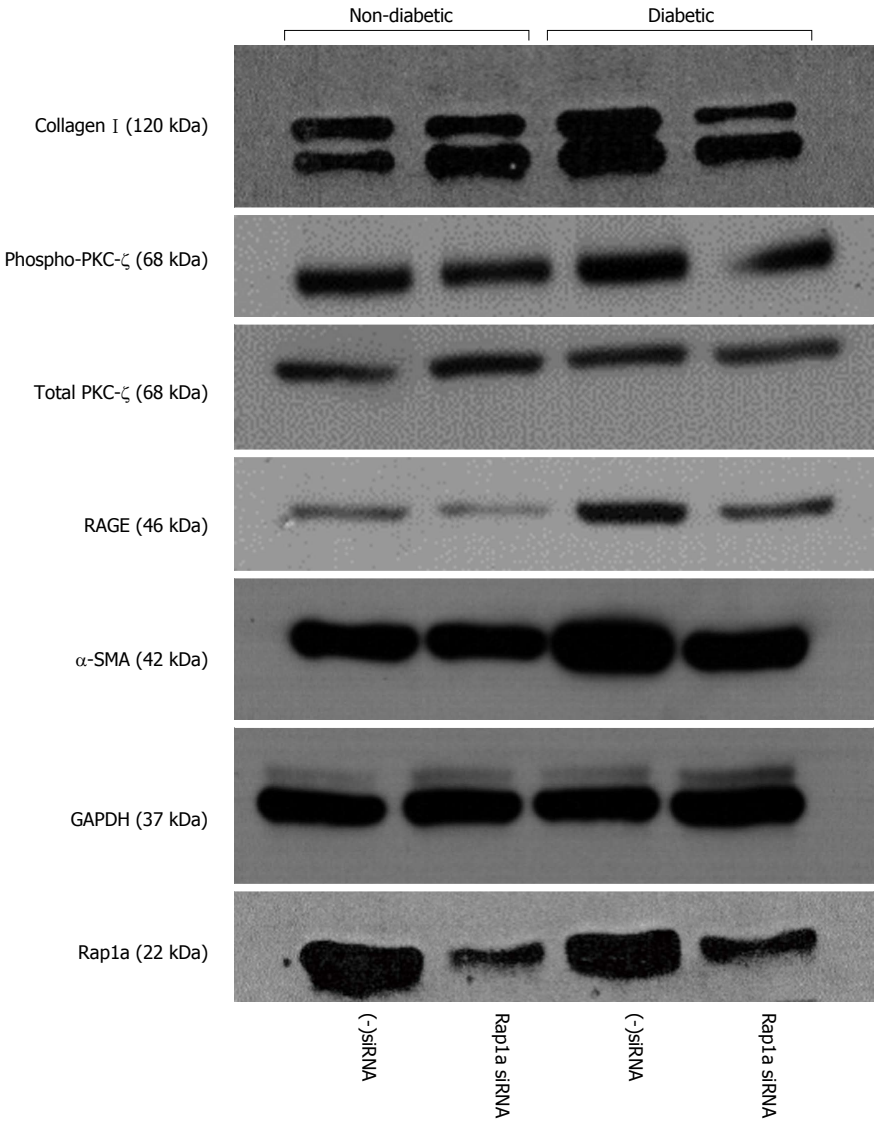

Figure 2 siRNA Rap1a knockdown in diabetic cardiac fibroblasts. Cardiac fibroblasts were isolated from age-matched 16 wk-old *db*/*wt* (non-diabetic) and *db*/*db* (diabetic) mice and using siRNA targeted to Rap1a silenced transcription and translation of Rap1a resulting in noticeable decreases not only in Rap1a expression, but also the downstream signaling outcomes RAGE, collagen I , phospho-PKC-ζ and α-SMA protein expression. RAGE: The receptor for the advanced glycation end product; PKC-ζ: The ζ isotype of protein kinase C; α-SMA: α-smooth muscle actin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

MAPK, involved in fibroblast growth factor-2 mediated angiogenesis were down regulated when Rap1a was depleted. Lastly, Jeyaraj *et al*[48] implicated Rap1a in roles that were intimately associated with the ECM remodeling process. Taken together, Rap1a and AGE/RAGE have been demonstrated to associate with increased myofibroblast formation and interstitial fibrosis independently. Figure 1 illustrates Rap1a's potential role in mediating the AGE/RAGE signaling pathway as discussed in the context of this review. While there is some evidence of a functional interplay between AGE/RAGE and Rap1a, the exact molecular interactions have not been fully characterized.

A series of studies by our laboratory suggest that Rap1a plays a role in fibrosis and myofibroblast differentiation in isolated diabetic and non-diabetic fibroblasts. Silencing Rap1a mRNA in diabetic fibroblasts returned profibrotic markers to nondiabetic levels. Isolated cardiac fibroblasts from 16 wk-old non-diabetic (heterozygous, wt/db) and diabetic (homozygous, *db*/*db*) mice were treated with siRNA targeted to Rap1a and a negative control of scrambled siRNA (data not shown) was used. 48-h post siRNA treatment, noticeable decreases were measured, not only in Rap1a expression, but also RAGE, collagen I , phospho-PKC-ζ, and α-smooth muscle actin protein expression (Figure 2). Inhibiting Rap1a protein expression down-regulated the molecular switch used to activate PKC-ζ to promote AGE/RAGE-mediated fibrosis. While these studies are still in a preliminary stage, we are working to expand our understanding of the significance of these alterations using not only siRNA technology, but also generating a double knockout mouse model to ascertain the role Rap1a plays in diabetic cardiomyopathy.

CONCLUSION

From the evidence that is presented, a cellular and mo-

lecular mechanism for Rap1a-mediated activation of AGE/RAGE-dependent myocardial remodeling exists. This review is the first of its kind to provide Rap1a as a unique target for therapeutic strategies aimed at reducing chronic hyperglycemia-mediated ECM production and accumulation in diabetic patients. While much still needs to be performed to increase our understanding of this causal relationship, our laboratory is working towards defining the signaling cascade involving Rap1a and PKA in the AGE/RAGE signaling cascade which ultimately mediates fibroblast myocardial remodeling. These studies provide insight into the inter-signaling components of this cascade that could ultimately help in reducing ECM production and accumulation during hyperglycemia in T2DM patients.

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Cardiac adipose tissue and its relationship to diabetes mellitus and cardiovascular disease

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Abstract

Type-2 diabetes mellitus (T2DM) plays a central role in the development of cardiovascular disease (CVD). However, its relationship to epicardial adipose tissue (EAT) and pericardial adipose tissue (PAT) in particular is important in the pathophysiology of coronary artery disease. Owing to its close proximity to the heart and coronary vasculature, EAT exerts a direct metabolic impact by secreting proinflammatory adipokines and free fatty acids, which promote CVD locally. In this review, we have discussed the relationship between T2DM and cardiac fat deposits, particularly EAT and PAT, which together exert a big impact on the cardiovascular health.

Key words: Epicardial adipose tissue; Pericardial adipose tissue; Type 2 diabetes; Cardiovascular disease

Core tip: Diabetes, a cardiovascular disease equivalent, has considerable effects on the cardiovascular system. Its impact works systemically, but may have more association with epicardial and pericardial adipose tissue locally at the level of the heart. These cardiac tissues have great interplay with diabetic patients and have potential to influence cardiovascular disease.

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INTRODUCTION

More than 25 million United States adults have type-2 diabetes mellitus (T2DM) and this figure will likely reach 50 million by 2050[1,2]. The relationship between metabolic diseases such as T2DM and regional fat deposits, particularly epicardial adipose tissue (EAT) and pericardial adipose tissue (PAT), play an important role in the development of cardiovascular diseases (CVD). Both EAT and PAT are a subset of visceral adipose tissue (VAT) associated with T2DM. They are metabolically active visceral fat deposits found around the heart[3], that are strongly associated with CVD including coronary artery disease (CAD) and the development of cardiac arrhythmias, predominantly due to the secretion of pro-inflammatory mediators and cytokines[4]. In this paper, we review the emerging evidence of impact of T2DM on VAT and the specific role of EAT and PAT both as a cardiac risk marker and as a potentially active player in the development of cardiovascular pathology.

RESEARCH

We searched MEDLINE and PubMed for original articles published between 1984 and 2014, focusing on epicardial adipose tissue and type 2 diabetes mellitus. The search terms we used, alone or in combination, were

"epicardial fat", "epicardial adipose tissue", "pericardial fat", "pericardial adipose tissue", "insulin resistance", "type 2 diabetes mellitus", "metabolic syndrome", "cardiovascular disease", "coronary artery disease", "congestive heart failure", and "atrial fibrillation", which yielded 121 articles. All articles identified were English-language, full-text papers and abstracts. We finally selected 87 articles, which were relevant to our current discussion.

T2DM AND CARDIAC VISCERAL FAT

Cardiac disease is the leading cause of death in T2DM, and many have sought to determine the mechanism of development of cardiac dysfunction[5]. Interestingly, diabetic patients with no evidence of CAD or hypertension have also been found with cardiac abnormalities, even when they are asymptomatic. Studies have shown that the metabolic derangements in T2DM primarily contribute to the cardiac problems[6], which, in part, are due to increase in visceral fat deposits and being frequently accompanied by disorders of glucose metabolism[7]. Obesity, specifically abdominal VAT, is an independent risk factor for CVD[8], and is prominent in patients with T2DM[7]. Moreover, studies have shown the correlation between excessive adipose tissue deposition and development of diabetes[9]. Central and VAT is associated with endocrine disorders due to the release of substances such as free fatty acids (FFA), leptin, adiponectin, pro-inflammatory agents, and decreased anti-inflammatory factors. As a result, it often results in unfavorable glucose metabolism and T2DM[10,11]. It has also been well demonstrated that pre-diabetic and diabetic patients are associated with significantly higher PAT burden compared to normoglycemic patients[12]. In a cross sectional study, the impact of obesity and T2DM on adipocytokines (adiponectin, leptin and resistin), inflammatory markers [tumor necrosis factor-α (TNF-α), Interleukin (IL)-6 and high sensitive C-reactive protein (HsCRP)] were evaluated[13]. Obesity was found to significantly lower adiponectin levels, while increasing leptin and IL-6 levels along with HsCRP. There is also a strong association between the increased expression of resistin, another adipocyte-secreted factor, and insulin resistance[14], with the burden of EAT volume being greater in individuals with metabolic syndrome, increased insulin resistance and diabetes mellitus[15,16], and is significantly higher in patients with T2DM than in non-diabetic subjects[4]. The serum profile of coronary artery bypass grafting patients showed significantly higher levels of HsCRP and lower levels of adiponectin compared to body mass index (BMI)-matched controls, supporting the role of VAT in causation of systemic inflammation[17]. Adiponectin has been shown to have a protective role with anti-inflammatory properties suppressing TNF-α and IL-6[13,18]. Hypoadiponectin levels in obesity along with elevated TNF-α, HsCRP and IL-6 were shown to correlate with insulin resistance seen in this population[13]. Interestingly leptin and resistin levels were not shown to consistently correlate with insulin resistance.

EAT and omental fat were shown to have broadly comparable pathogenic mRNA profile[17]. EAT and PAT are both forms of VAT, which store lipids and have demonstrated increased expression of the above mentioned hormones, chemokines and cytokines, with the addition of monocyte chemotactic protein-1 and IL-1β[19]. These adipokines also impair insulin-signaling pathways leading to insulin resistance and reduced nitric oxide (NO) synthesis, causing unopposed vasoconstriction[20]. Thus, the endocrine function of EAT and PAT play a significant role in patients with metabolic syndrome. In fact, the examination of EAT and PAT found that PAT is associated with VAT and metabolic syndrome features such as T2DM, than that of EAT[21]. On the other hand, EAT thickness showed independent positive correlation with metabolic parameters including postprandial glucose ($P = 0.049$), HbA1c level ($P < 0.001$), and homeostasis model assess of insulin resistance ($P = 0.047$)[22]. EAT accumulation was seen to strongly correlate with serum fibroblast growth factor 21, which is known to improve insulin sensitivity despite an increment in its serum levels in T2DM patients. Thus, excessive EAT in T2DM patients may exert bivalent, unfavorable and adaptive effects on progression of cardiovascular diseases[23].

In obese patients with T2DM, adipocytes from epicardial fat infiltrate the myocardium, which refers to a strong association of intra-myocardial fat content to the echocardiographic epicardial fat thickness. Similarly, EAT has been found to be significantly related to intra-abdominal visceral fat, suggested by echocardiographic studies[24,25], and PAT may increase up to 400 g in T2DM patients (with 100 g in healthy lean people)[26]. Yang *et al*[12] demonstrated the burden of PAT in diabetic and pre-diabetic subjects, revealing that PAT volume was much higher in pre-diabetics and diabetics as compared to normoglycemic subjects.

However, it is important to distinguish EAT and PAT from obesity-specific lipotoxic cardiomyopathy, in which excessive fat proliferates inside cardiac muscle causing left ventricular remodeling and eventually cardiomyopathy. This develops after subcutaneous adipose tissues and VAT are unable to accommodate the excess fat in the obese patients leading to intracellular accumulation of lipids and FFA, eventually forming myocardial steatosis[27].

ANATOMICAL, METABOLIC AND FUNCTIONAL DIFFERENCES BETWEEN EAT AND PAT

Epicardial and pericardial adipose tissue are close, however anatomically clearly different. EAT is not symmetrically distributed around the heart (Figure 1). EAT volume and thickness varies depending on the location (Figure 2). PAT (Figure 3) has a different embryonic origin than that of EAT as it originates from the embryonic primitive thoracic mesenchyme[24], and clinically are different. In

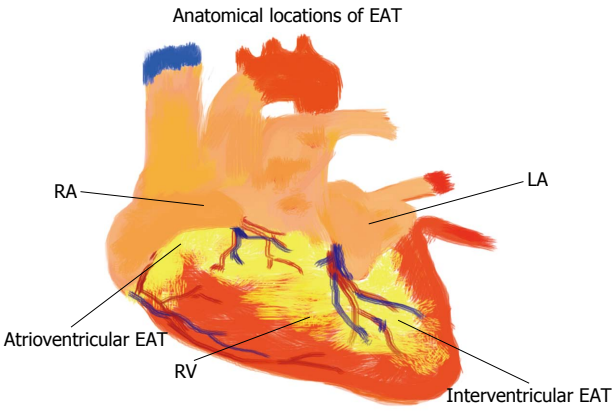

Figure 1 Anatomical locations of epicardial adipose tissue. RV: Right ventricle; RA: Right atrium; LA: Left atrium; EAT: Epicardial adipose tissue (yellow color refers to EAT).

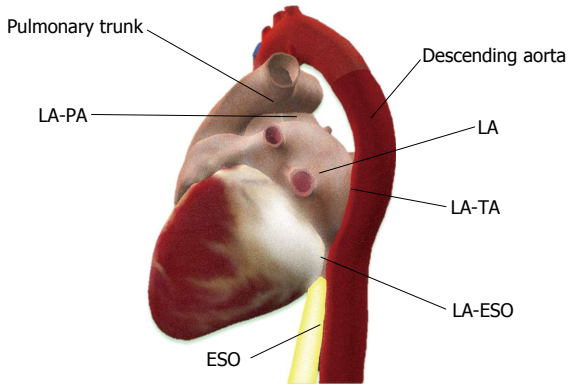

Figure 2 Periatrial epicardial adipose tissue around left atrium (heart in lateral axis view). LA-PA: Epicardial adipose tissue (EAT) between left atrium and pulmonary artery; LA-TA: EAT between left atrium and thoracic aorta; LA-ESO: EAT between left atrium and esophagus.

the existing literature, the terminologies have often been erroneously overlapped without clear differentiation between these two entities. Some suggest the use of a terminology, which encompasses three types of fat around the heart: epicardial, pericardial and paracardial fats. In this terminology, paracardial fat often refers to the fat located on the external surface of the parietal pericardium, while the term pericardial fat is used to represent EAT plus paracardial fat. It is important to be familiar with these terms to avoid confusion. In our opinion, it is rather more important to differentiate the "true pericardial fat" from "paracardial fat" as these two have different endocrine and metabolic properties. The true pericardial fat (epi-pericardial fat) should encompass the epicardial and pericardial fat (*i.e.*, fat located above the myocardium and up to the parietal pericardium; epicardial fat being located between the outer wall of the myocardium and the visceral layer of pericardium and pericardial fat being located between the visceral and the parietal pericardium), while paracardial fat should clearly be considered as the fat located outside the parietal pericardium.

EAT is a metabolically active visceral fat deposit found around the heart, between the pericardium and

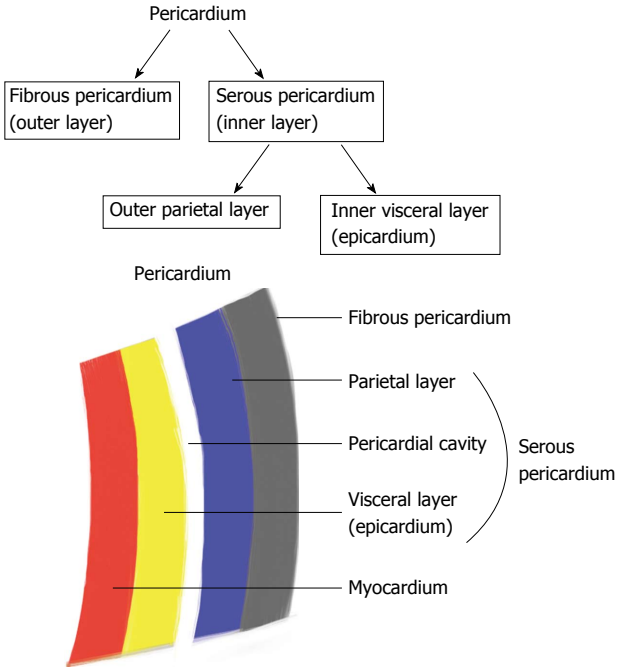

Figure 3 Pericardium/Pericardial layers.

myocardium[3]. EAT can be found in highest concentration in the atrioventricular and interventricular grooves and alongside the coronary arteries, and lesser so around the atria, over the free wall of the right ventricle and over the apex of the left ventricle. PAT may be defined as EAT plus paracardial fat, whereas paracardial fat is located on the external surface of the parietal pericardium within the mediastinum[28]. EAT varies from PAT and other local fat depots in the size of its adipocytes, where as epicardial adipocytes are smaller in size and high in number (high number of pre-adipocytes). The best imaging tool for quantification of both EAT and PAT remains uncertain. Their thicknesses and volumes can be evaluated by echocardiography, computed tomography (CT) or magnetic resonance imaging (MRI)[24,29]. Due to distinct attenuation values of fat on chest or cardiac CT and MRI, EAT and PAT are both readily identified with ability to calculate the tissue volume and thickness. Furthermore, MRI accurately correlates with EAT and PAT seen on echocardiography imaging[30].

Biochemically, EAT and PAT are different. Investigation into EAT and PAT suggests that these two tissues have different metabolic and physiologic properties[31]. Under physiological situations, EAT is cardioprotective which can be explained by its anti-atherogenic/anti-inflammatory properties, high FFA release and uptake and low glucose requirements, serving as a major source of energy to the heart and thermoregulatory properties[32]. It is also known to provide mechanical support to the coronary arteries as well as anti-toxic effects by protecting heart from high levels of FFA. In diabetics, lack of insulin impairs cardiac glucose transport and oxidation, resulting in FFA becoming the preferred means of energy supply[33]. To make available this increased requirement

Table 1 Studies showing the relationship between pericardial adipose tissue and epicardial adipose tissue and the development of coronary artery disease

Ref.	Year	Diagnostic modality	Results
Taguchi *et al*[86]	2001	Computerized tomogram	Pericardial fat was the strongest independent variable for severity of CAD, determined by coronary angiogram
Jeong *et al*[41]	2007	Echocardiogram	Epicardial fat thickness significantly correlated with the severity of CAD in patients with known CAD
Ahn *et al*[38]	2008	Echocardiogram	Epicardial adipose tissue was an independent predictor of CAD
Greif *et al*[36]	2009	Computerized tomogram	Patient with any coronary plaque showed a significantly higher pericardial adipose tissue volume compared to patients without coronary plaques
Shemirani *et al*[40]	2012	Echocardiogram	Confirms the presence of association between epicardial fat thickness and severity of CAD

CAD: Coronary artery disease.

of the heart for FFA, the diabetic heart upregulates its luminal lipoprotein lipase (LPL) activity, which can result in abnormal FFA supply and utilization by the heart tissue, potentially initiating cardiac dysfunction[33]. Importantly, EAT has low levels of LPL and acetyl-CoA as compared to subcutaneous fat[34], though the cardio-protective role of PAT is not clear[31]. Despite these protective qualities, EAT in excess can become cardio-toxic resulting in local inflammatory changes and cardiac dysfunction[32,35]. In non-diabetic patients with excessive EAT, the presence of fatty acid binding protein-4 in epicardial adipocytes, and its increased expression, promotes the development of metabolic syndrome[32] and T2DM.

CARDIAC ADIPOSITY, DIABETES MELLITUS AND CAD

PAT and EAT have firmly been recognized as a contributor to the development of CAD[36-41], and several cross sectional studies (Table 1) have shown similar results. PAT is emerging as a novel risk factor for CVD development[42] and progression[43], as CAD has been shown to correlate with PAT more consistently than other general measures of adiposity like body mass index or waist circumference[42]. PAT volume has been a predictor of increased death and disability for CVD[44], and independently linked with coronary artery calcification (CAC)[45]. EAT has also been shown to correlate with CAC[43] and has a statistically significant correlation between EAT and CAC in both diabetic and non-diabetic patients ($P = 0.01$, $r = 0.60$; $P = 0.02$, $r = 0.38$, respectively)[46]. The Multi-Ethnic Study of Atherosclerosis study showed a stronger correlation between PAT and the incidence of future coronary heart events in a group of patients without history of CAD, than that of other cardiac risk factors such as BMI or waist circumference[42].

EAT has been studied more extensively than PAT. EAT differs from PAT, not only in its location, but also by its blood supply. EAT derives its blood supply from coronary circulation, whereas PAT is supplied by non-coronary sources[32]. There is a functional and anatomic relationship between EAT and muscular components of the heart as these components share the same coronary blood supply, due to the lack of fascia separating the adipose tissue and myocardial layers[3]. Because of the highly metabolic paracrine and endocrine functions of EAT, it has been proposed to play a role in the pathogenesis of CVD by contributing to increased carotid intima media thickness (CIMT) in those with metabolic syndrome[47], CAD[37-41], increased left ventricle (LV) mass[48] and diastolic dysfunction[49,50]. The release of pro-inflammatory and pro-atherogenic factors into the circulation advancing CVD is more significantly linked to VAT accumulation, metabolic syndrome and other situations related to oxidative stress[32]. Pathophysiological effects of abnormal EAT may be explained by the expression of an enzyme-sPLA2-IIA which is generally found in human atherosclerotic lesions[32]. In patients with CAD, catalase levels in EAT are lower than in subcutaneous fat resulting in higher oxidative stress, which further contributes to atherosclerosis.

It is the close anatomical relationship between EAT and the coronary arteries, combined with its biologically active properties that participates in the pathogenesis of diabetic coronary atherosclerosis[4,51]. Iacobellis *et al*[52] demonstrated that the expression of anti-inflammatory and antiatherogenic properties of adiponectin was approximately 40% lower in the EAT of patients with CAD than in that of normal controls.

Apart from above, EAT was also shown to play an important role in the prediction of no-reflow phenomenon in ST elevation myocardial infarction treated with primary percutaneous intervention (PCI)[53]. The no-reflow was defined as < 70% ST-segment resolution following primary PCI. EAT has also been shown to be one of the independent factors associated with restenosis post-stenting warranting target vessel revascularization[54]. Smooth muscle proliferation, secondary to the local inflammatory mediators, have been postulated as mechanism of restenosis in this population[54].

EAT volume also has a significant role in promoting CVD and was shown to be positively and independently related to coronary atherosclerotic burden[55], and was significantly increased in patients with acute coronary syndrome[14]. Multivariate logistic regression analysis indicated that EAT thickness was an independent indicator for significant coronary artery stenosis after adjusting for traditional risk factors (OR = 1.403, $P = 0.026$)[22]

assessed by cardiovascular magnetic resonance imaging in asymptomatic T2DM patients. Echocardiographic measurement of EAT thickness ⩾ 7 mm was shown to identify individuals with higher probability of coronary atherosclerosis[56]. Furthermore, EAT thickness ⩾ 5 mm in general population may identify individuals with higher likelihood of detectable carotid atherosclerosis, but did not have any significant association with CIMT[57]. However, EAT thickness in patients with metabolic syndrome showed a linear positive correlation with CIMT[47]. Similar association was also found in human immunodeficiency virus receiving highly active antiretroviral therapy[58]. These studies establish that the correlation between EAT and CIMT is stronger in high-risk individuals prone to atherosclerosis than in the general population. It also demonstrates the existence of independent paracrine effects in addition to the endocrine effect, to account for the consistent association of EAT and coronary atherosclerosis[59].

CARDIAC ADIPOSITY AND VENTRICULAR FUNCTION

EAT and associated inflammatory cytokines, particularly hypoadiponectin levels and reduced NO synthesis, may have direct effect on myocardium causing dysfunction independent of ischemic pathophysiology[60]. PAT was shown to be significantly associated with LV diastolic dysfunction in people with CAD and normal ejection fraction independent of other risk factors including diabetes and hypertension[61]. Variation in regional fat distribution has been reported in patients on peritoneal dialysis[62]. Increased EAT thickness determined by echocardiogram in such patients was shown to be the most powerful determinant of LV diastolic dysfunction among other variables[63]. In addition to the paracrine metabolic effect as discussed earlier, mechanical effect of increased PAT has also been shown to contribute to the pathophysiology of diastolic dysfunction[63]. Additionally, patients with LV diastolic dysfunction had significantly increased EAT volumes[64].

On contrary, in patients with congestive heart failure (CHF) and severely reduced left ventricular ejection fraction (LVEF), EAT has been found to be significantly reduced[65]. LV function in such patients correlated best with EAT/Left Ventricular Remodeling Index ratio[65], raising a possible protective role of EAT to remodeling myocardium. Khawaja *et al*[66] demonstrated similar results with a stepwise decrease in EAT volume from controls to patients with moderate CHF (LVEF 35%-55%) and severe heart failure (LVEF < 35%). Though the paracrine metabolic effects and possible role as source of FFA to myocardium in demand has been postulated as mechanism for this correlation[65], the exact pathophysiology remains elusive. Further study is needed to access the possible confounding role of lipid lowering therapies to this finding in such patients.

CARDIAC ADIPOSITY, DIABETES MELLITUS AND ARRHYTHMOGENICITY

Obesity is a well-established risk factor for atrial fibrillation (AF), as altered atrial electrical function is considered an important mechanism for the relation of obesity and increased AF risk. Atrial tissue in diabetic subjects demonstrates persistent oxidative stress compared with nondiabetics; which can potentially play a role in the development of interatrial conduction delay[67]. Evidence on the impact of EAT thickness, particularly in the area of posterior left atrium, is associated with persistent AF[68,69]. PAT is also associated with a higher incidence of AF, both paroxysmal (OR = 1.11, 95%CI: 1.01-1.23, P = 0.04) and persistent (OR = 1.18, 95%CI: 1.05-1.33, P = 0.004), independent of other risk factors[69]. PAT's unique anatomic proximity to the myocardium and atrial conduction system may modify atrial electrophysiology and promote subsequent risk for arrhythmogenesis[70]. Based on PAT's influence on altered P-wave indices (PWI), potential mechanisms by which increases in PAT may lead to changes in atrial conduction include prolonged atrial depolarization, diminished voltage, and heterogeneous atrial activation related to fibrosis, hypertrophy, and fatty myocardial infiltration[70].

Two independent studies reported significant association of pericardial fat volume with AF both paroxysmal and persistent even after adjustment for traditional risk factors[69,71]. The possible mechanisms speculated were secondary to increase in left atrial size associated with pericardial fat[72,73] and local inflammatory effects induced by pericardial adipose tissue as discussed earlier *via* paracrine and endocrine route. This speculation was based on the evidence that systemic inflammation marked by CRP was associated with presence of AF and also predicted the patients at risk for future development of AF[74].

PWI and PAT were found to be associated independent of ectopic visceral and intra-thoracic fat depots[70], supporting the role of PAT in atrial conduction. Voltage-dependent PWI (P-Wave amplitude, P wave area and P wave terminal force) may be enhanced by hypertrophy of left atrium seen with pericardial fat. At the same time it may also be decreased due to fibrosis and effects on summation vector secondary to insulation effect[70]. The insulation effect does not affect the voltage-independent PWI (P wave duration and PR interval), however hypertrophy and fibrosis may still affect the conduction time[70]. P-wave terminal force is more closely associated with pericardial fat than other voltage-dependent PWI[70]. This is due to the fact that blocked posterior inter-atrial bundles seen with PAT causes anterior to posterior activation of left atrium resulting in a terminal negative deflection on the electrocardiogram in lead V1. PAT has been questioned to contribute to the P wave dispersion seen in obese individuals[71].

With further advancements in imaging, thickness of the posterior peri-atrial fat pad between left atrium

and the esophagus was found to correlate with the AF burden[68]. Their proximity to the pulmonary vein ostia would explain the correlation, as triggers for AF initiation are located in the pulmonary vein ostia[75]. EAT total and inter-atrial septal thickness was shown to be related to left atrial volume independently even after adjustment for other confounding factors[76]. PAT has also been associated with increased risk of AF recurrence after ablation[77]. PAT volume has also been identified as a novel risk factor for post-operative AF after coronary artery bypass grafting[78].

MANAGEMENT OF EAT AND PAT

As excessive cardiac adipose tissue have correlations with poor cardiovascular outcomes, research into possible reversal of the tissue has been studied. Weight loss through bariatric surgery and calorie restriction has shown a corresponding decrease in EAT volume and thickness. EAT thickness decreased in obese subjects who underwent an aggressive 6-mo long weight loss program (mean 20 kg) by adhering to a very low-calorie diet (900 kcal/d)[79]. Similarly, weight loss after bariatric surgery (average weight loss of 40 kg) was associated with a decrease in EAT thickness[80]. Conversely, the compared effects of pioglitazone and metformin treatment in T2DM patients demonstrated an increase in PAT volume in pioglitazone-treated patients after 24 wk[81]. Nonetheless, the correlation between increased cardiac adipose tissue has been associated with several features of metabolic syndrome, including fasting insulin[82]. Further studies are needed to show the effects of controlling these measures with changes in size of the cardiac adipose tissues.

CONCLUSION

Cardiac adipose tissue is metabolically active and associated with various metabolic derangements in the body leading to insulin resistance, atherosclerosis, metabolic syndrome and CVD. It has become clear that the adipose tissue around the heart is a critical indicator of CVD burden. Lifestyle and medical improvements may reduce this impact, as the evidence through the use of ultrasound has documented that weight loss is associated with a decrease in pericardial fat stores in both non-diabetic[79,83,84] and diabetic[85] subjects. In diabetics, metabolic derangements are significantly linked with cardiac adiposity, thus it should be considered screening for EAT or PAT as CVD risk factors in diabetic patients. Many aspects between EAT and PAT overlap. Clinicians and researchers must have a clear understanding of their physiological and pathological differences to expand on screening, managing and reducing the impact that EAT and PAT have on CVD.

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Intensive diabetes management and goal setting are key aspects of improving metabolic control in children and young people with type 1 diabetes mellitus

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Abstract

Diabetes control in children remains poor in spite of advances in treatment for last 10 years. The aim of this review was to look at various aspects of intensive therapy in the management of type 1 diabetes such as insulin regimes, role of target setting, psycho-educational approaches and self-management. To achieve good metabolic control, clear goal setting with adequate support for self-management are essential. Psycho-educational and behavioural interventions aimed at specific areas of management have shown significant improvement in quality of life and diabetes control.

Key words: Type 1 diabetes; Children; Metabolic control; Intensive; Management; Goal setting

Core tip: The aim of diabetes treatment is to maintain normoglycaemia in order to prevent long term complications. Insulin is the mainstay of diabetes treatment and is delivered by various regimens. Superiority of one regimen over the other is not established. Newer techniques with sensor augmented pumps have shown improvement in the diabetes control. Other aspects of intensive treatment are goal setting and adequate multidisciplinary support for self-management. Self-management is necessary to achieve the goals of diabetes treatment. Interventions based on clear psycho-educational principles are shown to be effective in improving outcomes.

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INTRODUCTION

Type 1 diabetes is characterised by autoimmune destruction of the β cells leading to insulin deficiency. It accounts for 90% of childhood diabetes in the western world. The incidence has been increasing over past 2 decades and poses a global challenge[1]. The aim of diabetes management in children is to achieve near normoglycaemia without major hypoglycaemic episodes and to prevent long term complications associated with hyperglycaemia[2].

Early normalisation of blood sugars with intensive insulin therapy might lead to improved long term control and higher endogenous insulin production 1 year after the diagnosis[3]. Good glycaemic control in patients with Insulin Dependent Diabetes mellitus delays the onset and slows the progression of long term complications. Several approaches are taken when aiming for low glucose targets. The Diabetes Control and Complication trial

Table 1 Review of studies comparing different insulin regimens

Ref.	Method/population	Outcome
de Beaufort *et al*[10]	Observational cross-sectional international study/2036 patients(11-18 yr)	No improvement in glycaemic control over a decade Those on twice daily free mix had significantly better control and the ones on twice daily injections had the worst HbA1c
Holl *et al*[11]	Multicentre Observational study/872 patients (11-18 yr)	Deterioration in metabolic control in all three groups over 3 yr period One group had moved from twice daily to multiple injections
Haller *et al*[12]	Observational Study (enrolled patients were on preferred regimes from 12 paediatric endocrinologists)/229 patients (9-15 yr)	Increased number of insulin types correlated with increased HbA1c
Nordly *et al*[13]	Multicentre cross sectional sudy/874 (< 16 yr)	Children with 2 injections a day had significantly better control than children on 3 or four injections a day
Paris *et al*[14]	Multicentre cross-sectional study/2743 patients (< 20 yr)	Insulin pump users had better control. No difference between MDI or 2-3 injections a day
Jakisch *et al*[15]	Multicentre matched pair cohort analysis, comparing CSII to MDI/434 matched pairs	Significantly better HbA1c in CSII group after 1 yr but subsequently no difference at 3 yr

MDI: Multiple daily insulin; CSII: Continuous subcutaneous insulin infusion; HbA1c: Hemoglobin A1c.

(DCCT) clearly showed that intensive therapy aiming for lower target blood sugars measured by lower mean glycosylated haemoglobin A1c (HbA1c) reduced the risk for onset and progression of diabetes complications[4]. However, intensive treatment does not just include intensive insulin regimes but patient education, counselling and effective diabetes self-management[5]. It can best be provided with well-sourced multidisciplinary team with focus on treatment goals and regimes, self-management, patient education and frequent clinic visits[6]. There is considerable diversity in delivery of these interventions and it has been a challenge to find practical, clinic based interventions that can provide improvement in HbA1c similar to those achieved in DCCT. Hvidoere study group have demonstrated that the clinical and metabolic goals or targets are more important in determining the outcomes than the therapeutic regimen on its own. Self management, structured education for the patient and family, and close telephone contact with the diabetes team are also associated with reduced hospitalisations and emergency room visits[7].

The purpose of this review is to examine the key aspects of improving metabolic control in children and young people with diabetes who have characteristics and needs that dictate different standards of care. We will look specifically at the impact insulin delivery and regime, self-management of diabetes which includes psychological intervention, self-education programmes and goal setting in improving outcomes.

INSULIN DELIVERY AND REGIME

Treatment with insulin is the mainstay of therapy in type 1 diabetes mellitus. Many formulations are available but with the advent of newer analogues, they are mainly used in treatment in children. There is no data on the long term benefits of these analogues but they provide more flexibility and some improvement in the care of diabetes[8,9].

The choice of insulin regime depends on the individuals The basal bolus therapy or multiple daily insulin (MDI) regimes consists of long or intermediate acting insulin is given once or twice a day with boluses of rapid acting insulin analogue with meals. Insulin pump or continuous subcutaneous insulin infusion (CSII) works on similar principles but delivers short acting analogue continuously with boluses at meal times. After DCCT trial, these modalities have become the norm of diabetes treatment. Other methods include use of pre-mixed insulin which contain fixed ratio mixtures of short and intermediate acting insulins. They are given as two injections a day. Currently, there is no clear evidence that one insulin regime is superior to other on its own[10].

There are various cross-sectional studies looking at different insulin regimes (Table 1) but none of them have found any clear evidence that one is superior over the others.

Insulin pumps

There are several systemic reviews and meta-analysis including a Cochrane review comparing CSII to MDI[16]. Most of them have favoured CSII for better control but recent meta-analysis comparing CSII to MDI showed no significant change in HbA1c from baseline level after 16 wk or more of follow up in children. Overall CSII has been found to yield better quality of life compared to MDI, however benefit to glycaemic control is variable[16,17].

Sensor augmented pump therapy (SAP) which integrated CSII with a continuous glucose sensor. In a comparative meta-analysis sensor-augmented insulin pump use resulted in a statistically and clinically significant greater reduction in HbA1C levels than with MDI or self-monitoring of blood glucose (SMBG) in persons with type 1 diabetes mellitus[17]. Sensor-Augmented Pump Therapy for A1C reduction. STAR 3 study has shown that compared to MDI, SAP offers rapid glycemic advantage in children and adolescents which lasted for the entire year of study phase[18,19].

SMBG is the key to achieving main goals of insulin therapy. Several studies have established that frequency

of SMBG is directly proportional to improved HbA1c levels[12,20].

More recently continuous glucose monitoring (CGM) has been used and can provide information on trends of blood glucose levels. It is considered to be useful for children with poorly controlled diabetes. Recent Cochrane review has shown that there is limited evidence of improved glycaemic control in patients with poorly controlled diabetes. But the review found larger decline which was statistically significant in HbA1c for real-time CGM users starting on insulin pump therapy(sensor augmented pumps) compared to patients using MDI and SMBG (conventional therapy)[21].

GOAL SETTING AND PSYCHOLOGICAL INTERVENTIONS TOWARDS SELF MANAGEMENT

Specific goal setting is an encouraging way of improving adherence to diabetes management in young people[22]. As parental support and involvement is associated with better management of diabetes in children and adolescents, their perception of goals for optimal management of diabetes is associated with actual control achieved in children[23]. Hvidoere study group has documented persistent inter-centre differences in the mean HbA1c over 10-year period in spite of changes to the insulin regimes[10]. They concluded that target setting might me the most influential factor in lowering the HbA1c[24]. Key findings from their work suggests that best metabolic results are obtained by physicians who target driven and teams and families have unanimity of purpose[7].

It is important to have necessary self-management skills in order to achieve goals of diabetes therapy. Diabetes self-management is the process of providing the person with diabetes education, knowledge and skills needed to successfully manage diabetes[25]. It is multi-dimensional and refers to the young persons or/and parents sharing responsibility and decision making for achieving optimal control[26]. Goals for self management varies considerably by age, development, family characteristics, duration of diabetes and lifestyle[27,28]. Adolescence could be a challenging time in control of diabetes. It has been recognised that diabetes control tend to decline during this period[29]. As young people strive for autonomy, social influence and peer pressure with desire to fit in can be higher priority than diabetes management for some young people[30,31]. Various psychological and educational interventions are used to empower the young person with necessary self-management skills but efficacy of one over another is not established. Wysocki *et al*[32] found that youths with suboptimal pre-treatment status with high autonomy to maturity (AMR) did better with intensive treatment over 18 mo period compared to the ones who had low AMR and better HbA1c. An integrated review in 2011 demonstrated that there is a clear relationship between self-management and metabolic control but there is multitude of factors playing part[28].

Research has also shown that there is an association between psychosocial factors and metabolic control in a large international cohort of adolescents with type 1 diabetes mellitus[33] Good metabolic control is associated with better quality of life in adolescents[34,35]. It is also associated with families of children with better control reporting lower disease burden. Behavioural interventions for young people with diabetes and their parents have demonstrated improvement in adherence of treatment[36]. Interventions based on clear psycho-educational principles are most effective[37]. In a systematic review of psychological interventions for improving diabetes control, psychological therapies led to significant improvement in glycaemic control in children and adolescent compared to adults[38]. A case study of 9 adolescents with consistently poor control previously has shown has shown marked improvement with coaching[39]. These findings show that assessment of psychosocial factors should be an integral part of the paediatric diabetes care in this population[33,40].

There are various structural education programmes for adults with type 1diabetes which have shown improvement in their control as well as quality of life[41,42]. However, there is need for practical, clinic based educational interventions for children and adolescents. Various trials have reported disappointing outcomes in improving control when applied to families and children in a real life setting[43,44]. The Kids in control of food is a structured education course based on Dose Adjustment for Normal Eating course which is a current adult education programme. The pilot showed significant improvement in quality of life and self-efficacy at 3 and 6 mo. There was no change in glycaemic control overall but improvement trend in those with poorest control[45]. Results of the randomised trial will hopefully give us more information on the effect of highly structured group education on a population with wide range of glycemic control[46].

In a systematic review by Hampson *et al*[37], it was concluded that educational and psychological interventions are most likely to be effective if demonstrate an interrelatedness of various aspects of diabetes management. There is a gap in evidence as no complete understanding of where these interventions to be targeted.

CONCLUSION

Good metabolic control is needed to prevent long term complications of diabetes. It is challenging in the paediatric population to achieve optimal control due to various developmental and psychological factors[47]. Psycho-educational and behavioural interventions play an important role in the diabetes management. However, there is need for practical, cost effective interventions which could be applied to the diabetes population in a clinic setting such as goal setting and psychosocial interventions. Svensson *et al*[48] have reported significant improvement in diabetes control independent of number of injections per day or insulin regimens but thought to be due to increased focus

on treatment goals, glucose monitoring and optimising care in their population over 10 years period. Overall, this review concludes that clear goal setting with good multidisciplinary team effort working together with families and children towards specific targets may be the key to good diabetes control.

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MINIREVIEWS

Diagnosis of hepatic glycogenosis in poorly controlled type 1 diabetes mellitus

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Abstract

Hepatic glycogenosis (HG) in type 1 diabetes is a underrecognized complication. Mauriac firstly described the syndrome characterized by hepatomegaly with altered liver enzymes, growth impairment, delay puberty and Cushingoid features, during childhood. HG in adulthood is characterized by the liver disorder (with circulating aminotransferase increase) in the presence of poor glycemic control (elevation of glycated hemoglobin, HbA1c levels). The advances in the comprehension of the metabolic pathways driving to the hepatic glycogen deposition point out the role of glucose transporters and insulin mediated activations of glucokinase and glycogen synthase, with inhibition of glucose-6-phosphatase. The differential diagnosis of HG consists in the exclusion of causes of liver damage (infectious, metabolic, obstructive and autoimmune disease). The imaging study (ultrasonography and/or radiological examinations) gives information about the liver alterations (hepatomegaly), but the diagnosis needs to be confirmed by the liver biopsy. The main treatment of HG is the amelioration of glycemic control that is usually accompanied by the reversal of the liver disorder. In selected cases, more aggressive treatment options (transplantation) have been successfully reported.

Key words: Hepatic glycogenosis; Type 1 diabetes mellitus; Hepatomegaly; Glycogen; Glucose transporters; Insulin; Glucokinase; Glycogen synthase; Glucose-6-phosphatase

Core tip: This review contain an extensive revision of the case reports described in literature; in particular glycemic control (elevation of glycated hemoglobin, HbA1c levels, presence of ketoacidosis and insulin dosage), imaging studies and bioptic findings are summarized and discussed. The pathophysiological mechanisms behind the accumulation of glycogen in hepatocytes in patient with poorly controlled type 1 diabetes mellitus are described in detail.

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INTRODUCTION

Primary glycogenosis or glycogen storage disease is a well known hereditary disease affecting liver and muscles, characterized by the presence of hepatomegaly, hypoglycemia, muscle weakness and growth delay. On the contrary, secondary glycogenosis [hepatic glycogenosis (HG)] is less described in the literature, but it may be frequently

observed and underrecognized in type 1 diabetes (T1D)[1]. Mauriac[2] firstly described the syndrome in 1930. The main features in prepuberal children are hepatomegaly with increased liver enzymes, growth impairment, delay puberty and Cushingoid features in poorly controlled T1D[3]. In young adults with T1D the syndrome is uncomplete, and, in fact, only hepatomegaly with increased liver enzymes are present. The latter alterations are often underrecognized or confused with fatty liver disease or non-alcoholic steatohepatitis (NASH), that is common in T2D[4]. In rare cases, glycogen storage hepatomegaly has been described also in T2D[5].

PATHOPHYSIOLOGY

As pointed out by Wasserman[6], 4 grams of glucose circulates in the blood (a small fraction of the body mass) and 100 grams of glycogen are present in the liver. In glucose homeostasis, the liver plays a significant role for synthesis, storage and redistribution of carbohydrates, with opposite effects during hyperglycemic (glucose uptake and glycogen synthesis) and hypoglycemic conditions (glycogenolysis and gluconeogenesis)[7].

The glucose transport into cells is mediated by fourteen members of membrane glucose transporter (GLUT) molecules, divided into three families (Classes 1 to 3). The expression of the GLUTs varies between different cellular subtypes in liver (hepatocytes, endothelial cells, Kupffer cells and cholangiocytes)[8].

The liver is not considered as an insulin-sensitive tissues, such as skeletal and cardiac muscle, brown and white adipose tissue and endothelial cells. In fact, the transport of glucose into the hepatocytes is mainly mediated by the GLUT2 (insulin-independent, low-affinity, high-capacity with a Km of 10-20 mmol/L), but hepatocytes also express lower levels of GLUT1, GLUT3, GLUT4 (insulin-dependent), GLUT8, GLUT9, GLUT10[9-16] (Figure 1).

After the entrance, glucose is available for the intracellular metabolism. Glucokinase is a phosphorylating enzyme, acting with not stringent substrate specificity for glucose (it is able to phosphorylate hexoses like mannose or fructose in addition to glucose), to produce glucose-6-phosphate (G6P)[17]. There are four mammalian isoenzymes (hexokinases I -Ⅳ or A-D), displaying extensive sequence identities[18]. Glucokinase (GCK, or hexokinase Ⅳ or D) has a low affinity for glucose ($S_{0.5}$ approximately equal to 6 mmol/L) and a rate of reaction with sigmoid dependence on intracellular glucose concentration (cooperativity), operating as an ultrasensitive physiological glucose sensor in hepatocytes with non-limiting glucose transport. If blood glucose is below 5 mmol/L (90 mg/dL) there is no significant effect of GCK on G6P production and subsequent steps, ensuring that hepatic glycogen synthesis is only engaged when blood glucose levels are high.

In the human liver, expression of GCK is strictly dependent on the presence of insulin, and the sterol regulatory element binding protein (SREBP1c), a master regulator of lipogenic enzymes, has been proposed to be a mediator of insulin induction of GCK[19].

Moreover, the GCK activity is modulated by the GCK regulatory protein (GCKRP) that binds and inhibits GCK, competitively with respect to glucose[20]. GCK is localized to the nucleus of the hepatocyte, where it is retained by GCKRP, but moves into the cytosol when glucose levels increase.

The hydrolysis of G6P to glucose (the inverse reaction of GCK) is mediated by the enzyme glucose-6-phosphatase (G6Pase), and its deficiency causes the impaired glycogenolysis of one type of the genetic accumulation of glycogen in hepatocytes, previously described by Von Gierke [glycogen storage disease type I (GSD1a)][21,22]. GSD1a has typical hypoglycemic events after a four to six hour fast (differentiating GDS1a from T1D), lactic acidosis, hypertriglyceridemia, and hyperuricemia[23].

The G6P is successively converted into G1P by phosphoglucomutase. Then, uridine diphosphate (UDP)-glucose pyrophosphorylase transforms G1P into UDP-glucose in the presence of uridine triphosphate, releasing inorganic pyrophosphate.

The G6P, after the phosphorylation by GCK, functions as an allosteric activator of the phosphorylated glycogen synthase (GS) for the glycogen synthesis[24]. Insulin significantly stimulates the glycogen synthesis in hepatocytes. Insulin binds the α-subunit of insulin receptor (IR) on the cellular surface of hepatocytes, inducing the dimerization of the α2β2 complex and the tyrosine kinase activity of the β-subunits. Then, the IR is autophosphorylated and the IR activation recruits and phosphorylates several substrates, including insulin receptor substrate 1-4. The downstream signaling proteins activates phosphotidylinositide-3-kinase (PI3K) to protein kinase B (PKB, also known as Akt signaling cascade), a pathway controlled *via* a multistep process[25]. In particular, the activation of PI3K converts phosphatidylinositol (3,4)-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The 3-phosphoinositide-dependent protein kinase 1 and 2 (PDK1 and PKD2) phosphorylate and activate PKB/Akt, allowing to bindPIP_3 at the plasma membrane. The activation of PKB/Akt phosphorylates and inhibits glycogen synthase kinase 3 (GSK3). GSK3 is a negative regulator of GS, through the phosphorylation at COOH-terminal residues. The result of insulin signal transduction is the GS dephosphorylation that activates the enzyme and the glycogen production. The GS is the rate-limiting enzyme for glycogen synthesis and it catalyzes the addition of α-1,4-linked glucose units from UDP-glucose to a nascent glycogen chain[26]. The UDP-glucose is the glycosyl donor in the reaction catalyzed by GS. There are two GS isoforms: the muscle GS (encoded by *GYS1* gene), and the liver isoform (encoded by *GYS2* gene)[27].

Glycogen is a branched polymer of glucose residues connected by α-1,4-glycosidic linkages formed by the enzyme GS and branchpoints formed *via* α-1,6-glycosidic linkages, introduced by the branching enzyme, occurring

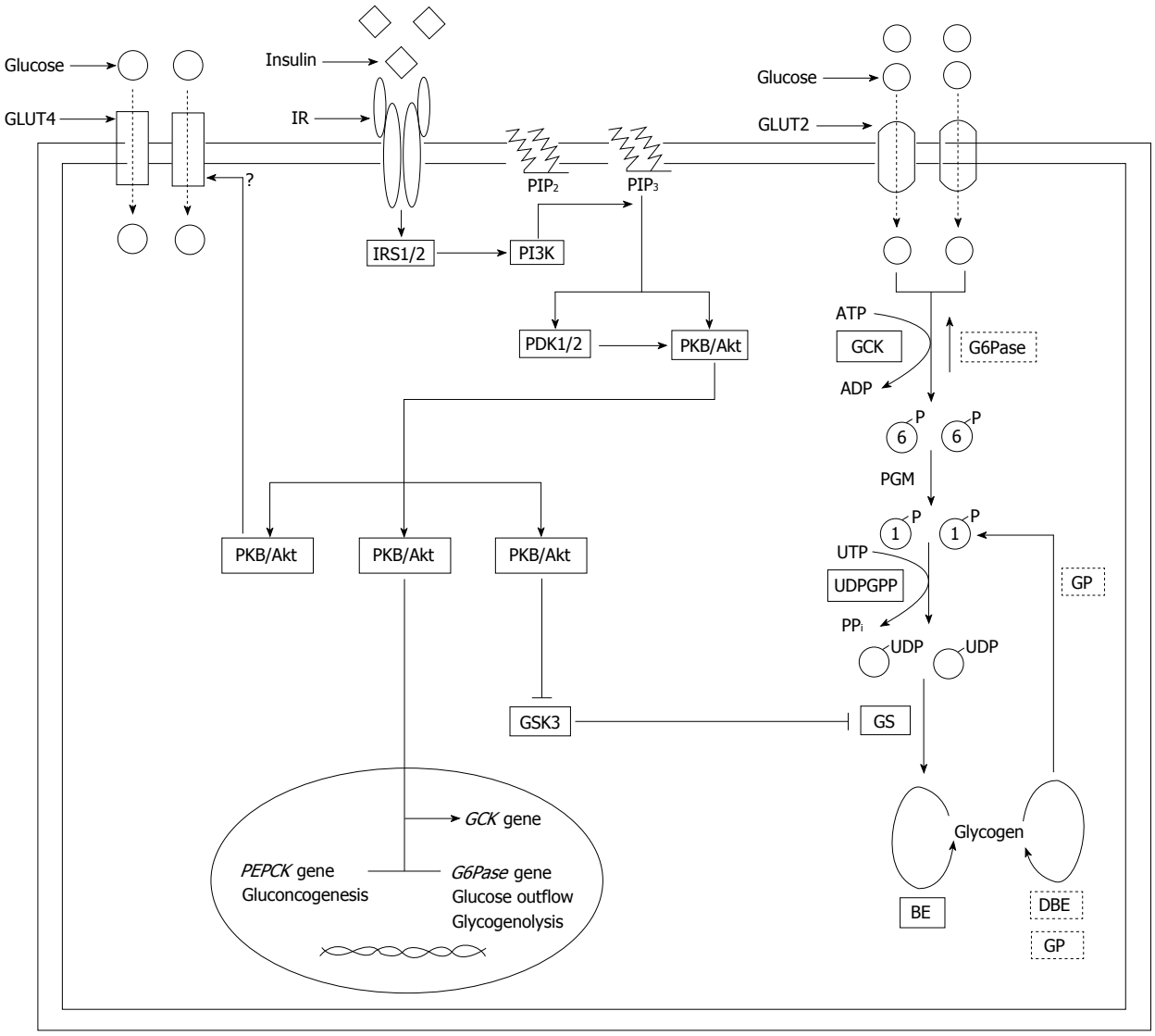

Figure 1 The metabolic pathways of glycogen synthesis in hepatocytes. GLUT: Glucose transporter; IR: Insulin receptor; PIP_2: Phosphatidylinositol (3,4)-bisphosphate; PIP_3: Phosphatidylinositol (3,4,5)-trisphosphate; IRS: Insulin receptor substrate; PI3K: Phosphotidylinositide-3-kinase; PDK1/2: 3-phosphoinositide-dependent protein kinase 1 and 2; PKB/Akt: Protein kinase B; GCK: Glucokinase; G6Pase: Glucosio-6-phosphatase; PGM: Phosphoglucomutase; UDPGPP: UDP-glucosepyrophosphorylase; GP: Glycogen phosphorylase; GSK3: Glycogen synthase kinase 3; GS: Glycogen synthase; PEPCK: Phosphoenolpyruvate carboxykinase; BE: Branching enzyme; DBE: Debranching enzyme; UTP: Uridine triphosphate; PPi: Pyrophosphate.

every 8-12 glucose units.

New glycogen synthesis begin near the plasma membrane, at the periphery of the hepatocyte. Then, glycogen deposits grow from the periphery towards the interior of the cell. Through this way of glycogen deposition, hepatocytes may store large amounts of glycogen.

Glycogen degradation takes place in the reverse order. Glycogen phosphorylase (GP) is the key enzyme in glycogenolysis, yielding G1P[28]. When hepatocytes are depleted of glucose, the GP-mediated phosphorolysis of glycogen proceed from the interior to the exterior of the hepatocyte[29]. Phosphorylase kinase stimulates GP and protein phosphatase 1 inhibits phosphorylase kinase and GP.

Besides stimulating the glycogen synthesis, insulin severely inhibits hepatic glucose output, suppressing gluconeogenesis and glycogenolysis, by inhibiting expression and activity of the key enzymes phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase[30].

The inhibition of gluconeogenesis and glycogenolysis are IR-mediated PI3K and Akt dependent effects. Akt translocates into the nucleus, where it phosphorylates FOXO1 (a member of the O-class of forkhead/winged helix transcription factors), inhibiting *PEPCK* and *G6Pase* gene transcription[31]. Moreover, Akt phosphorylates and inhibits CRTC2, cAMP response element binding protein-regulated transcription coactivator-2, also reducing hepatic gluoconeogenesis[32].

Adolescent diabetic patients with their metabolic activity, dietary intake, and disease state (high frequency of ketoacidosis and increase in exogenous insulin) represents a high-risk subjects, with diabetes control often deteriorating[33].

In T1D patients with poor glycemic control, two

Table 1 Summary of hepatic glycogenosis in type 1 diabetes patients

Ref.	Sex	Age (yr)	BMI	AST (U/L)	ALT (U/L)	HbA1c (%)	Insulin (U/kg)	Glucose (mg/dL)	US exam	CT scan	Biopsy
[51]	M	16	20	66	58	11.1	0.98	198	X		X
[52]	F	17		138	164	12			X		X
[54]	M	19		262	519	12.7[a]				X	X
[55]	F	19	27	98	49	7.9			X	X	X
	M	37		769	844	16			X	X	X
[56]	F	19	23		800	12.2[a]			X		X
[57]	F	3		300	350	9.5[a]	1.5	522	X		No
	M	16		100	200		1.3	810	X		No
[33]	M	14		290	127	13.4	1.6		X	X	X
	F	17		102	147	13.3[a]	1.8				X
	F	16		567	316	12.2[a]			X		No
[1]	F	17	21.4	1620	629	13	0.9		X		X
[58]	M	16	21.1	578	526	11.0[a]					X
[59]	F	22	18.6	1028	365	13.8			X		X
	F	26	23.6	914	307	12.9			X		X
	F	20	21	1310	346	13.6			X		X
[53]	F	29		4000	1900	15.3[a]			X		X
[60]	M	13			1000	13	1.2		X	X	X
[36]	F	20		249	383	13.3[a]				X	X
[61]	F	13			113	8.8[a]		890	X		X
[35]	F	19		83	97	[a]		520			X
	M	12		47	49	13.5[a]		635			X
	F	22		77	48			183			X
	M	8		H	H						X
	F	15		N	N						X
	M	22		360	1100	16.0[a]		404			X
	M	25		1128	1629	10.8					X
	M	16		H	H	[a]					X
	M	20		120	N	9.9		288			X
	F	18		57	N	10.8		137			X
	M	28		1544	1099			H			X
	M	34				10		259			X
	M	16		1354	1413			365			X
	F	23		224	255						X
[41]	F	19			199	14.6[a]				[b]	X

[a]Recent ketoacidosis; [b]Magnetic resonance imaging. H: High level; N: Normal levels; M: Male; F: Female; BMI: Body mass index; AST: Aspartate-aminotransferase; ALT: Alanine-aminotransferase; HbA1c: Glycated hemoglobin; US: Ultrasound; CT: Computed tomography.

combined events are usually present, promoting hepatic glycogen deposition: hyperglycemia (as pointed out by increased blood glucose level and glycated hemoglobin, HbA1c) and consequent large amount of insulin (as demonstrated by elevated insulin dose as UI/kg of body weight/day). In hyperglycemia, glucose passively enters the hepatocytes by insulin-independent GLUT2, and it is rapidly phosphorylated, with inhibition of its release from hepatocytes[34]. The GCK convert the glucose into the G6P, with subsequent trapping in the hepatocyte. Then, an increased insulin administration promotes the polymerization of G6P in glycogen by GS, driving the large amount of glycogen synthesis in the presence of high cytoplasmic glucose concentrations[29]. Therefore, glycogen is trapped within the hepatocytes as a result of a combination of both hyperglycemia and insulin treatment. The consequent liver damage become evident with the blood release of aminotransferases.

Repeated ketoacidosis episodes in T1D increase the risk for hepatic glycogen overload, since diabetic ketoacidosis (a fatal complication of poor controlled diabetes) is usually treated with sustained levels of intravenous insulin (in the presence of high glucose blood concentrations).

DIAGNOSIS

Nowadays Mauriac syndrome during childhood is uncommon especially with the advent of new insulin analogues and intensive insulin regimens. More frequently, patients affected are teenager or young adults and the diagnosis may be difficult[3]. During adulthood, the key symptoms are hepatomegaly, abdominal pain, and other symptoms such as nausea and vomiting. Laboratory findings are high levels of glucose, glycated hemoglobin (HbA1c, demonstrating a poor long-term glycemic control) and aminotransferases [aspartate and alanine, Aspartate-aminotransferase (AST) and Alanine-aminotransferase (ALT), respectively, suggesting liver damage][35]. The range of AST/ALT values is from 47/48 UI/L to 4000/1900 UI/L (Table 1). The investigations about hepatomegaly and elevated aminotransferases include investigations for infectious diseases, metabolic (such as Wilson disease), obstructive or oncologic causes and autoimmune liver tests to exclude all these possible

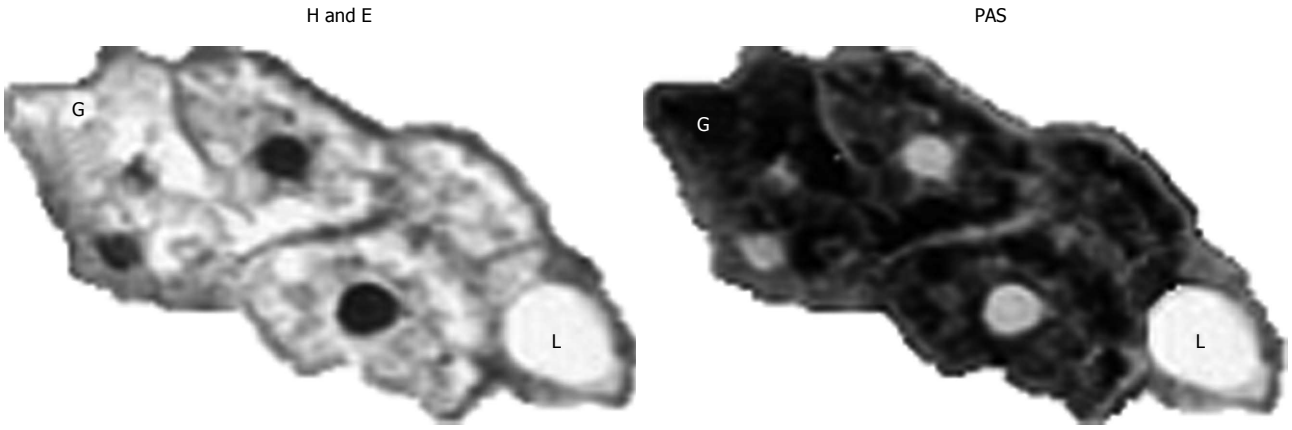

Figure 2 Schematic reproduction of staining with Hematoxilyn and Eosin *vs* Periodic Acid Schiff. The glycogen (G) disappears in H and E whereas it stains (red) in PAS. The presence of lipids (L) in focal vescicular steatosis is demonstrated by lack of staining both in HE and PAS. H and E: Hematoxilyn and Eosin; PAS: Periodic Acid Schiff.

causes and make the differential diagnosis[33]. The ultrasonographic examination of the liver is a simple and useful procedure to have information about the dimension and the characteristics of the liver tissue[34]. In few cases, T1D patients were submitted to an abdomen computed tomography scan. Unfortunately, HG cannot be clinically distinguished from non-alcoholic fatty liver disease or non-alcoholic steatohepatitis (NASH) by history, physical examination or ultrasound: the gold standard examination is the liver biopsy[36]. The preparation of the tissue is very important for the identification of the glycogen in tissue sections. The Carnoy's solution is rapid acting, gives good nuclear preservation, retains glycogen and dissolves lipids[37]. The cytoplasmic swelling due to glycogen can be quickly demonstrated by the staining with Best's carmine or periodic acid-Schiff (PAS) with and without diastase since the slides treated with diastase, that digest the glycogen, lack the PAS positive staining[34]. The main histological features of HG are marked glycogen accumulation leading to pale swollen hepatocyte, no or mild fatty change, no or minimal inflammation, no or minimal spotty lobular necrosis, and intact architecture with no significant fibrosis[35]. Best's carmine is another common used stain for glycogen, that appears bright red in sections. On the contrary, in hematoxylin & eosin sections, pale hepatocytes loose their glycogen during tissue preparation and may give a hint to hepatic glycogenosis (Figure 2)[37].

Navigator-gated and gradient-echo shimmed point-resolved spectroscopy with proton hydrogen1 (1H) magnetic resonance (MR) has been recently proposed to quantify liver glycogen concentrations in vivo, even if this measurement is more challenging than just lipid quantification[38]. In previous studies, an MR technique was used with (1-^{13}C) glucose to measure changes in net hepatic glycogen concentration in normal and diabetic subjects[39,40].

To our best knowledge, in only one study the authors investigated the liver by the means of the MR imaging, with anatomical purposes[41].

Whereas it is well known that glycogen storage diseases, particularly type I , develop hepatic adenoma that potentially progress into hepatocellular carcinoma (HCC), to our best knowledge no data have been published about the association of diabetic glycogenosis and the progression of carcinogenesis to HCC[42-47].

TREATMENT

The more the T1D patients (and their caregivers) obtain a good glycemic control, the more HG is expected to be minimal.

The Diabetes Control and Complication Trial (DCCT) is a well-known multicenter randomized trial that compared intensive with conventional therapy in insulin-dependent diabetes mellitus, demonstrating a prevention of diabetic complications[48]. The percentage of adolescent (13-18 years old) was 9%-19% of 1441 patients, with a 2.6-8.9 years of disease duration, a starting insulin dose of 0.62-0.72 U/kg of body weight/day and an insulin dose after 5 year of 0.46-1.10 U/kg of body weight/day[48,49].

As it has been described in the literature, the mean insulin dose in T1D patients with HG was significantly higher than in DCCT trial (1.33 U/kg), having been treated with supra-physiologic doses of insulin (Table 1).

Repeated ketoacidosis episodes in T1D significantly increase the risk for hepatic glycogen overload, since diabetic ketoacidosis (a fatal complication of poor controlled diabetes) is usually treated with sustained levels of intravenous insulin (in the presence of high glucose blood concentrations). As matter of fact, a high percentage of the HG cases described in the literature presented diabetic ketoacidosis, with a frequency of about 40% (14/35 cases), confirming the association of sustained insulin treatment and the development of HG.

With a significant difference from NASH, HG is completely reversible with a good metabolic control[50,51]. Adequate management of glucose and insulin levels can result in complete remission of clinical, laboratory and histological abnormalities[52]. Continuous subcutaneous insulin infusion should be considered as an option because the insulin requirements usually come down with improved

glycemic control[41]. In severe and rare cases, pancreatic transplantation has been reported to be effective[53].

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MINIREVIEWS

Type 2 diabetes mellitus and Alzheimer's disease

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Abstract

Epidemiological and biological evidences support a link between type 2 diabetes mellitus (DM2) and Alzheimer's disease (AD). Persons with diabetes have a higher incidence of cognitive decline and an increased risk of developing all types of dementia. Cognitive deficits in persons with diabetes mainly affect the areas of psychomotor efficiency, attention, learning and memory, mental flexibility and speed, and executive function. The strong epidemiological association has suggested the existence of a physiopathological link. The determinants of the accelerated cognitive decline in DM2, however, are less clear. Increased cortical and subcortical atrophy have been evidenced after controlling for diabetic vascular disease and inadequate cerebral circulation. Most recent studies have focused on the role of insulin and insulin resistance as possible links between diabetes and AD. Disturbances in brain insulin signaling mechanisms may contribute to the molecular, biochemical, and histopathological lesions in AD. Hyperglycemia itself is a risk factor for cognitive dysfunction and dementia. Hypoglycemia may also have deleterious effects on cognitive function. Recurrent symptomatic and asymptomatic hypoglycemic episodes have been suggested to cause sub-clinical brain damage, and permanent cognitive impairment. Future trials are required to clarify the mechanistic link, to address the question whether cognitive decline may be prevented by an adequate metabolic control, and to elucidate the role of drugs that may cause hypoglycemic episodes.

Key words: Dementia; Alzheimer; Type 2 diabetes; Aging; Cognitive decline; Mild cognitive impairment; Insulin; Hypoglycemia; Hyperglycemia

Core tip: Epidemiological and biological evidences support a link between type 2 diabetes (DM2) and Alzheimer's disease (AD). Persons with diabetes have increased incidence of cognitive decline and AD. Increased cortical and subcortical atrophy is present after controlling for vascular disease and inadequate cerebral circulation. Recent studies confirmed the role of insulin as possible link between DM2 and AD. Altered insulin signaling may contribute to AD biochemical and histopathological lesions. Hyperglycemia and hypoglycemia also have deleterious effects on cognitive function. Future trials would clarify the mechanistic link, and if cognitive decline may be prevented by an adequate metabolic control, and avoiding hypoglycemia.

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INTRODUCTION

Type 2 diabetes mellitus (DM2) and Alzheimer's disease (AD) are age-related conditions, both characterized by increased incidence and prevalence with aging[1,2].

DM2 is one of the fastest growing epidemics at present, which is frequently associated with aging. Characteristic features of DM2 include impairments in insulin actions

and signaling. Insulin resistance in peripheral tissues results in hyperglycemia and hyperinsulinemia. AD is the most common neurodegenerative disorder, and its incidence increases with age[3]. AD is characterized by the presence of several pathological hallmarks including neuronal loss, formation of senile plaques composed by extracellular deposits of amyloid beta, intracellular neurofibrillary tangles composed of aggregated hyperphosphorylated tau proteins in brain, proliferation of astrocytes, and activation of microglia. These features are accompanied by mitochondrial dysfunction and alterations in neuronal synapses[3]. The molecular and pathophysiological mechanisms that underlie AD still have many dark sides. Although etiology and the exact mechanism that trigger the pathological alterations of AD are still not clear, most studies have suggested that the deposit of the toxic amyloid-beta peptide caused by an abnormal processing of amyloid-beta precursor protein (amyloid cascade hypothesis), may initiate and/or contribute to the pathogenesis of AD.

EPIDEMIOLOGICAL EVIDENCES

Mounting epidemiological and biological evidences support a link between these two aging related diseases. First and foremost, diabetes mellitus is associated with changes in cognition, and cognitive dysfunction.

Persons with diabetes have been reported to hold a higher incidence of cognitive decline and AD; DM2 has been strongly associated with an increased risk of developing all types of dementia, including AD[2,4-6]. A systematic review including fourteen eligible longitudinal population-based studies of variable methodological quality found that in most studies the incidence of "any dementia" was higher in persons with diabetes than in those without diabetes[7]. Although, in some studies there are methodological limitations, the association remains strong. Some studies have relied on self-reported diagnosis of diabetes, and in the elderly population many patients with diabetes may remain undiagnosed. For the same reason, the duration of diabetes is also difficult to ascertain in older adults[8].

In a longitudinal cohort study, lasting up to 9 years, the risk of developing Alzheimer's disease was 65% higher in persons with diabetes than in non-diabetic controls[9]. In a community-based controlled study (Mayo Clinic Alzheimer Disease Patient Registry) the prevalence of diabetes and glucose intolerance was examined in patients with AD *vs* control participants without AD. The study suggested that frank diabetes (35%) or glucose intolerance (46%) might be present in up to 80% of patients with AD[10].

Even with the limitations discussed above, several studies have suggested that longer diabetes duration is generally associated with a higher risk for developing dementia[6,11,12]. In random effects models, DM2 was associated with lower levels of global cognition, episodic, semantic and working memory, and visuospatial ability at baseline[9]. Cognitive deficits in DM2 mainly affected the areas of psychomotor efficiency, attention, learning and memory, mental flexibility, and speed and executive function[13,14].

Recent studies have also shown a positive association between DM2 and mild cognitive impairment (MCI), and an accelerated progression from MCI to dementia in DM2[15]. A retrospective case-notes review of people with known diabetes who were resident in nursing homes in England showed very significant levels of disability and comorbidity, and in this setting, dementia was the most common comorbidity[16].

PHYSIOPATHOLOGICAL LINK

The strong epidemiological association has suggested the existence of a physiopathological link. However, the determinants of the accelerated cognitive decline in DM2 are less clear. The most studied hypothesis proposes that the primary cause of the association may be linked to the diabetic vascular disease and inadequate cerebral circulation, with subsequent silent ischemic damage induced by diabetes. However, even after controlling for cardiovascular risk factors, several studies on the cerebral structure of patients with diabetes have evidenced increased cortical and subcortical atrophy, besides increased leukoaraiosis, which were associated with impaired cognitive performance[17,18].

Most recent studies have focused on the possible role of insulin, and insulin action. Insulin resistance has been strongly implicated as a possible link between DM2 and AD. A condition of hyperinsulinemia, regardless of the presence of DM2, appears to be associated with a worse cognitive performance. There is a rapid growth in the literature pointing toward insulin deficiency and insulin resistance as mediators of AD-type neurodegeneration. De la Monte has even suggested that AD may be termed as "type 3 diabetes", indicating that AD may represent a form of diabetes that selectively involves the brain with molecular and biochemical features that overlap with diabetes mellitus[19].

The importance of the role of insulin in brain aging has long been known. Insulin has significant neurothrophic properties in the brain. The hormone is rapidly transported to the level of the central nervous system through the blood-brain barrier by a transport mechanism mediated by insulin receptors. It is interesting to note that these receptors are mainly localized at the level of the hippocampus, entorhinal cortex and frontal areas known to be involved in functions such as memory and learning. Insulin is also involved in the production of important neurotransmitters such as acetylcholine and norepinephrine. It is known that an acute increase in circulating levels of insulin, as it occurs in the post-prandial period, determines a physiological parallel increase of the concentrations of the hormone in the brain. A state of chronic hyperinsulinemia, as it occurs in insulin-resistance conditions and in DM2 may

determine a down-regulation of the insulin receptors at the blood-brain barrier, thus reducing the transport of insulin in the brain. Evidence is growing to link an alteration of metabolism and the deposition of precursors of amyloid in the brain that may occur in persons with diabetes, which is suggested as the pathogenesis of AD in DM2. The amyloid precursor protein is a transmembrane protein consisting of 770 amino acids; it is known to be the precursor of the amyloid beta involved in the etiopathogenesis of AD. Although the role of amyloid beta and its isoforms has yet to be elucidated, it seems to take part in numerous physiological processes. How can clinical hyperinsulinemia be a risk factor for AD even if insulin is an important neurothrophic factor? These two apparent paradoxal findings may be reconciled by the notion of insulin resistance. Whereas insulin is a neurothrophic factor at moderate concentrations, hyperinsulinemia with elevated concentrations of insulin in the brain may be associated with reduced amyloid-beta clearance due to competition for their common and main degrading mechanism-the "Insulin-Degrading Enzyme" (IDE). Insulin modulates metabolism of amyloid precursor protein decreasing intracellular accumulation. Insulin is degraded by the IDE, which is also involved in the metabolism and degradation of amyloid beta. This multifunctional enzyme degrades insulin and amylin, peptides related to the pathology of DM2, together with amyloid-beta peptide in the AD brain. Hyperinsulinemia may elevate amyloid beta through insulin's competition with amyloid beta for IDE[20]. Therefore, it has been suggested that the link between hyperinsulinemia and AD may be the IDE. Since IDE is much more selective for insulin than for amyloid beta, brain hyperinsulinemia may deprive amyloid beta of its main clearance mechanism, favoring its accumulation in the brain, and its consequent neurotoxic effects[21].

Disturbances in brain insulin signaling mechanisms represent early and progressive abnormalities and could account for the majority of molecular, biochemical, and histopathological lesions in AD. Increasing insulin resistance and hyperinsulinemia were associated with more hippocampal and amygdalar atrophy on magnetic resonance imaging (MRI) in persons with DM2 when compared to matched non-diabetic controls, regardless of vascular pathology[13,17]. Given these links, it has been suggested that may be a common underlying mechanism predisposes to amyloid deposition in the brain and in the pancreatic islet[10].

Glucose levels itself are a risk factor for cognitive dysfunction and dementia. In a prospective, community-based cohort study, higher plasma glucose concentrations were associated with an increased risk of dementia in populations with and without diabetes, suggesting that higher levels of glucose may have deleterious effects on the aging brain[22].

Although there is still limited knowledge concerning the association between impaired fasting glucose and/or impaired glucose tolerance and cognitive impairment, there is increasing evidence that these prediabetic conditions may increase the risk of AD in elderly patients. The risk of incident dementia increased in diabetic and in non-diabetic persons according to the average glucose concentrations during the preceding 5 years[22]. Hyperglycemia and hyperinsulinemia may accelerate brain aging also by inducing *tau* hyperphosphorylation and amyloid oligomerization, as well as by leading to widespread brain microangiopathy. Persons with diabetes are more prone to develop accelerated leukoaraiosis (white matter high-intensity lesions)[23].

GLYCEMIC CONTROL AND THE ROLE OF HYPOGLYCEMIA

The effect of diabetes treatment and glycemic control on dementia risk are less clear. It has been suggested that glycemic control may have a role in preserving cognitive performance among patients with DM2. Using baseline cognitive measures collected in the Memory in Diabetes, sub-study of the Action to Control Cardiovascular Risk in Diabetes trial, the authors found that a 1% higher glycated hemoglobin A (HbA1c) value was associated with a significant lower test performance and memory score in patients with diabetes[24].

HbA1c was also identified as an additional risk factor for a greater rate of brain atrophy. Enzinger *et al*[25], measuring the annual brain volume changes over 6 years with MRI in 201 participants in the Austrian Stroke Prevention Study, found significant differences in brain atrophy rates by quartiles of HbA1c levels[25]. Clustering of factors associated with the so-called metabolic syndrome in persons with high HbA1c suggests a link between this syndrome, which is associated with insulin resistance and hyperinsulinemia, with late-life brain tissue loss[25]. In diabetic patients, an inverse relationship was found between serum HbA1c and working memory, executive functioning, learning, and complex psychomotor performance, supporting the hypothesis that an inadequate glucose control may be associated with worsening cognitive function[26,27].

However, an excessively tight glycemic control in older persons with DM2, and its related increased risk of hypoglycemia, may also have deleterious effects on cognitive function[28]. In the presence of hypoglycemia, several responses occur within the brain, including activation of the central sympathetic nervous system; hypoglycemic symptoms include alterations of cognitive function, such as difficulty in concentrating and drowsiness, among others. Recurrent symptomatic and asymptomatic hypoglycemic episodes have been suggested to cause sub-clinical brain damage, and permanent cognitive impairment[29]. In addition, hypoglycemic states may increase the action of the receptors through an arteriolar vasodilatation. Since chronic hyperglycemia in DM2 is associated with endothelial alterations[30], this may cause in case of hypoglycemia a reduced vasodilating effect at the level of the blood-brain barrier, with a possible amplification of the brain

damage due to hypoglycemia itself. Among older patients with type 2 diabetes, a history of severe hypoglycemic episodes collected and reviewed using hospital discharge and emergency department diagnoses from 1980-2002 was associated with a greater risk of dementia[31]. More recently, a 12 years prospective population-based study of 783 older adults who were participating in the Health, Aging, and Body Composition Study, found a bidirectional association between hypoglycemia and dementia[32]. During the 12-year follow-up period, the participants who experienced at least one hypoglycemic event had a 2-fold increased risk for developing dementia, while older adults with DM2 who developed dementia had a greater risk for having a subsequent hypoglycemic event compared with participants who did not develop dementia[32].

Therefore, it has been suggested that drugs that cause lower postprandial glucose excursions and minor risk of hypoglycemia may prevent cognitive decline in older diabetic persons[33]. This data needs to be confirmed by future trials.

RESEARCH AND CLINICAL IMPLICATIONS

Cognitive function has not been included as an outcome in large scale randomized controlled trials of type 2 diabetes, and screening for dementia and cognitive impairment is still not included in routine diabetic patient care. There are sufficient epidemiological and clinical data to include an evaluation of cognitive complications in the clinical practice of persons with diabetes, in particular in those older than 70-75 years, and those with a long lasting history of diabetes.

There are some barriers in implementing a screening and diagnostic program for dementia in patients with diabetes. Neurocognitive testing in which an expert examiner administers a battery of tests to assess different aspects of cerebral function is still the gold standard for the diagnosis of dementia[14], and a computed tomography (CT) scan or an MRI may be required. This evaluation requires substantial financial and human resources. Screening cognitive tests are time consuming and CT scans are expensive[34]. However, diagnosis is even more important in older populations, because many older persons with diabetes nowadays live alone and self-manage their drugs. A mistake due to cognitive impairment may be extremely dangerous in particular in patients who need insulin, and self-practice insulin injections. Many hypoglycemic episodes may be due to errors in self-administration in undiagnosed subclinical demented patients.

CONCLUSION

There is convincing epidemiological evidence showing an increased risk of dementia in people with diabetes, but there are few mechanistic studies that provide a clear pathophysiological link, although the cause may be multifactorial. Cerebrovascular alterations, insulin action, insulin resistance, altered amyloid metabolism, chronic hyperglycemia, and recurrent hypoglycemic episodes seem to play a major role. Future trials are required to clarify the mechanistic link and to address the question whether cognitive decline may be prevented by an adequate metabolic control, and to better define the role of drugs that may cause hypoglycemic episodes. Clinicians treating older persons with diabetes should start to routinely search for cognitive impairment as well as they search for cardiovascular, renal, or other common complications of diabetic disease. There is sufficient evidence to support the view that time is probably arrived to incorporate cognitive evaluation in future national and international diabetic guidelines.

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MINIREVIEWS

Sirtuins as novel players in the pathogenesis of diabetes mellitus

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Abstract

Diabetes mellitus (DM) is a systemic and complex disease with micro and macrovascular complications that result from impaired metabolic pathways and genetic susceptibilities. DM has been accepted as an epidemic worldwide during the last two decades. A substantial gap in our knowledge exists regarding the pathophysiology of this metabolic disorder despite the improved diagnostic tools and therapeutic approaches. Sirtuins are a group of NAD^+ dependent enzymes that are involved in cellular homeostasis due to their deacetylating activity. In the present review, we aimed to discuss the role of associated sirtuins in the pathogenesis and treatment of diabetes mellitus.

Key words: Diabetes mellitus; Sirtuins; Hyperglycemia; Hyperlipidemia; Resveratrol

Core tip: Diabetes mellitus has been accepted as an epidemic worldwide during the last two decades. Despite the diagnostic tools and therapeutic approaches, the pathophysiology of this metabolic disorder and cellular defensive mechanisms are unknown. The maintenance of cellular homeostasis requires a well-organized network between glucose, amino acid and lipid metabolism. Sirtuins are a group of NAD^+ dependent proteins that are involved in cellular homeostasis due to their deacetylating activity. Of these, sirtuin 1, -3 and -4 have been the most extensively investigated. In the present review, we aimed to discuss the role of associated sirtuins in glucose and lipid metabolism and in the pathogenesis and treatment of diabetes mellitus.

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INTRODUCTION

Diabetes mellitus is one of the leading causes of cardiovascular morbidity and mortality despite the emergence of new diagnostic tools and therapeutic applications in clinical practice[1]. According to American Diabetes Association data, there are 17.5 million diagnosed and 6.6 million undiagnosed diabetics in the United States[2]. Hence, diabetes and its complications represent a significant economic burden. Hyperglycemia, insulin resistance, advanced glycation end products, polyol, hexosamine and protein kinase C pathways collectively contribute to the classical pathogenesis of diabetes complications. However, to date, we know that only serum glucose control is not sufficient to overcome the major cardiovascular (CV) events[3,4]. In this regard, novel risk factors including adipokines such as adiponectin, apelin, obestatin, leptin and resistin, chronic inflammation, and the renin-angiotensin-aldosterone system were found

Table 1 The characteristic features of sirtuins

Sirtuin group	Enzyme localization	Enzyme activity
SIRT1	Cytoplasm and nucleus	Deacetylase
SIRT2	Cytoplasm and nucleus	Deacetylase
SIRT3	Cytoplasm, mitochondrion and nucleus	Deacetylase
SIRT4	Mitochondrion	ADP-Ribosyl transferase
SIRT5	Mitochondrion	Deacetylase
SIRT6	Nucleus	Deacetylase and ADP-Ribosyl transferase
SIRT7	Nucleus	Deacetylase

SIRT1: Sirtuin 1.

to be involved in the pathogenesis of diabetes and its chronic complications[5]. It would be wise to search the main mechanisms of these undesirable pathophysiologic events responsible for increased CV morbidity and mortality in diabetic patients. In addition, treatment of these various entities separately is illogical. Therefore, the main pathogenetic mechanisms should be determined and new therapeutic agents should be identified to treat diabetes.

Mammalian sirtuins are a group of proteins that include seven NAD^+ dependent enzymes with homology to the silent information regulator 2 (Sir2) family of *Saccharomyces cerevisae*[6]. Activation or deactivation of the enzymes occur as a consequence of this deacetylation. Since both carbohydrate and lipid metabolism are affected in diabetes, it would be wise to consider that sirtuins may be the responsible key proteins that fight against the detrimental effects of these disorders. With this background, in this review, we sought to highlight the role of sirtuins as novel players in the pathogenesis of diabetes mellitus.

GENERAL FUNCTIONS OF SIRTUİNS IN CELLS

The main function of sirtuins is to deacetylate the important proteins for cellular homeostasis that regulate a wide variety of processes regarding protein, carbohydrate and lipid metabolism, mitochondrial homeostasis and programmed cell death mechanisms such as apoptosis and autophagy[7]. Sirtuins remove the acetyl groups from lysine residues of transcription factors, histones, specific enzymes including manganese superoxide dismutase and peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α) and other miscellaneous proteins that have important roles in cellular homeostasis[8]. As a consequence of the deacetylation, nicotinamide and 2'-0-acetyl-adenosine di phosphate (ADP) ribose are generated[9].

Experimental data showed the beneficial effects of decreasing food intake by 30% without malnutrition, also named calorie restriction (CR), on aging that could be mediated by sirtuin overexpression and this effect leads to increasing lifespan[10]. Increased intracellular NAD^+ concentrations and CR are the main effectors that can stimulate sirtuin activation. In energy rich conditions, NAD^+ is reduced to nicotine-amide adenine di nucleotide (NADH) and the proportion of NAD^+ to NADH is reduced during glycolysis, cyclic acid cycling, lipid β-oxidation and protein catabolism[11]. Two main sources of NAD^+ are the salvage pathway of nicotinamide catalyzed by the enzyme, nicotinamide phosphoribosyltransferase, and *de novo* synthesis from tryptophan metabolism[12].

Recent experimental studies showed that sirtuins can be found and activated in kidney, liver, spleen, lung, heart, muscle, brain, testis, ovary, thymus, pancreas, white and brown adipose tissue[13]. The localization of Sirtuin (SIRT) proteins differ and matter in the cell, hence, the different localizations develop various physiologic and possibly pathologic metabolic effects under certain stress conditions. SIRT1 resides both in the nucleus and cytoplasm and SIRT2 is primarily found in the cytoplasm, however, it can be transferred into the nucleus in a cell cycle-dependent manner. SIRT3, -4 and-5 exist in the mitochondrion. The last two members of the SIRT protein family, SIRT6 and-7 are found in the nucleus and the nucleolus of the cell, respectively[14]. Table 1 summarizes the characteristic features of sirtuins.

SIRT1 is the most studied member of the sirtuins, probably because of its generalized effects on the cell cycle, mitochondria metabolism, energy homeostasis, inflammation, oxidative stress and apoptosis[15]. SIRT1 can directly deacetylate nuclear histone proteins that results in repression of gene transcription[16]. On the other hand, the metabolic effects of SIRT1 depend on the deacetylation of non-histone proteins such as insulin receptor substrate 2, PGC-1α, peroxisome-proliferator-activated receptor (PPAR)-α, PPAR-γ, mitochondrial uncoupling protein 2 (UCP-2), liver X receptor, farnesoid X receptor and sterol-regulatory-element binding protein[17-21]. Due to its deacetylation activity, SIRT1 regulates insulin secretion, adipogenesis and myogenesis.

In contrast to other sirtuins, SIRT4 has an additional ADP-ribosyltransferase activity that is also involved in telomere maintenance, genomic stability and longevity[22,23].

SIRT5 is a mitochondrial sirtuin. The main activity of SIRT5 is translocating SIRT3 to the nucleus[24].

SIRT6 has auto-ADP-ribosyltransferase activity[25] and its main function includes genomic stability of cells in terms of DNA repair and modulating telomere maintenance[26].

THE ROLES OF ASSOCIATED SIRTUINS IN GLUCOSE METABOLISM AND DIABETES MELLITUS

Sirtuins, especially SIRT1, influence many steps of glucose metabolism in liver, pancreas, muscle and adipose tissue (Figure 1). The main regulator of these reactions is the deactylated form of PGC-1α in SIRT1 activated states[27].

Forkhead box group O (FOXO), a group of transcriptional factors, can sense nutrient deprivation and

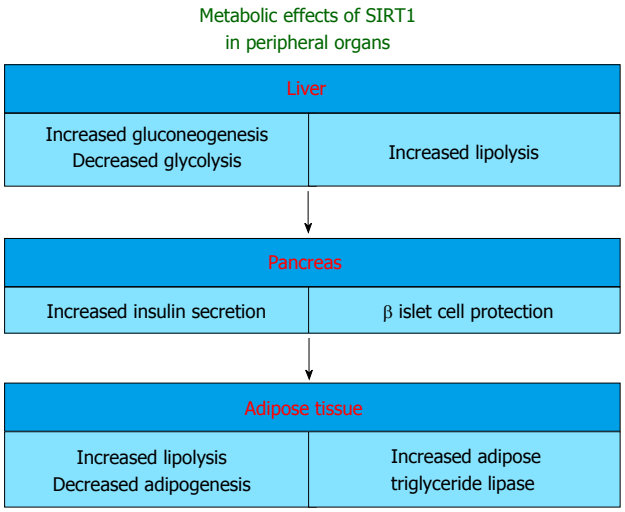

Figure 1 Metabolic effects of sirtuin 1 in peripheral organs.

promote cellular homeostasis[28]. FOXO1 regulates glucose metabolism[29] and feeding behaviors[30]. During the fasting state, the balance between insulin and glucagon (decreased insulin *vs* increased glucagon) stimulates gluconeogenesis *via* cAMP response element-binding protein regulated transcription coactivator 2 and FOXO1[31,32].

The link between FOXO proteins, Signal transducer and activator of transcription 3 (STAT3) and SIRT1 regarding hepatic glucose metabolism has been identified. FOXO1,-3a,-4 were found to be closely associated with increased expression of gluconeogenesis genes and decreased expression of glucokinase[33,34]. SIRT1 also regulates gluconeogenesis *via* deacetylation and thereby deactivates STAT3 which can inhibit the transcription of gluconeogenic genes in normal conditions[35].

The role of sirtuins in the pancreas has been demonstrated. Experimental data of SIRT1 overexpression suggested that serum insulin and cholesterol were diminished along with a reduction in adipose tissue volume and decreased obesity-induced insulin resistance[36,37]. Recently, beside experimental data, Song *et al*[38] also observed that adipose tissue SIRT1 may play a key role in the regulation of whole body metabolic homeostasis, and downregulation of SIRT1 in visceral adipose tissue may contribute to the metabolic abnormalities that are associated with visceral obesity in diabetic and obese women. SIRT1 deficient mice also exhibit low levels of serum glucose and insulin[39]. Despite the repetitive results of the studies regarding the CR induced SIRT1 expression, Moynihan *et al*[21] demonstrated that increased dosage of mammalian Sir2 in pancreatic beta cells enhanced glucose-stimulated insulin secretion in mice. Bordone *et al*[39] also pointed out that insulin secretion was reduced in SIRT1 knock-out mice and in pancreatic β islet cell lines in which SIRT1 had been knocked down by RNA interference. This effect partially depends on the SIRT1-mediated inhibition of UCP-2 in pancreatic islet β-cells[21]. UCP-2 is a mitochondrial inner membrane protein that regulates mitochondrial ATP synthesis. SIRT1 knock-out mice exhibit increased UCP-2 in β-cells along with low levels of serum insulin[39]. Increased pancreatic secretion of insulin and ATP were also demonstrated in UCP-2 knock-out mice[40]. In light of these studies, SIRT1 might be a positive regulator rather than a supressor of insulin in the postprandial fed state.

Insulin sensitivity is considered to be an important part of glucose metabolism. Protein tyrosine phosphatase 1B (PTP1B) is involved in glucose metabolism and diet-induced obesity[41]. PTP1B which is a tyrosine phosphatase for the insulin receptor, can be repressed *via* deacetylation. In accordance, resveratrol, an activator of SIRT1 may also inhibit PTP1B. Thus, SIRT1 might improve insulin sensitivity in insulin-resistant conditions by reducing PTP1B activity[42].

SIRT2 is a cytosolic deacetylase which was originally identified as a tubulin deacetylase. It was subsequently demonstrated that SIRT2 can also transiently shuttle into the nucleus in a cell cycle-dependent manner[43]. It is possible that besides their tubulin deacetylating function, nuclear proteins may be another target of SIRT2. In addition, researchers showed that SIRT2 was prominently expressed in adipocytes[44]. Krishnan *et al*[45] also found that SIRT2 was predominantly localized to the nucleus in adipocytes. PGC-1α has been strongly associated with energy expenditure[46]. The acetylation of PGC-1α has been reported to be critical in regulating its activity. In this regard, SIRT2 was found to deacetylate PGC-1α. The identification of PGC-1α as a SIRT2 substrate suggests that SIRT2 regulates adipocyte mitochondrial activity. Additionally, SIRT2 can deacetylate FOXO1 and FOXO3. Hence, SIRT2 was found to be closely associated with DNA repair, cell cycle, metabolism, apoptosis, and aging[47]. It has also been demonstrated that SIRT2 may increase the expression of the antioxidant mitochondrial superoxide dismutase due to its ability to deacetylate FOXO3 and consequently increase FOXO3 DNA-binding activity[48].

SIRT3 has beneficial effects on glucose metabolism by increasing insulin sensitivity and decreasing serum glucose. Hirschey *et al*[49] showed that high-fat diet feeding induces hepatic mitochondrial protein hyperacetylation in mice and downregulation of the major mitochondrial protein deacetylase SIRT3. They concluded that increased obesity, insulin resistance, hyperlipidemia, and steatohepatitis were prominent in mice lacking SIRT3 compared to wild-type mice. The same group also identified a single nucleotide polymorphism which encoded a point mutation in the SIRT3 protein. In this regard, impaired mitochondrial protein acetylation and polymorphism of SIRT3 have been shown to be closely associated with the metabolic syndrome[49].

Another important sirtuin involved in glucose metabolism is SIRT4. One of the target enzymes of SIRT4 is glutamate dehydrogenase (GDH) which converts glutamate to α-ketoglutarate in the mitochondrion[50]. SIRT4 inhibits amino-acid induced insulin secretion by repressing GDH[51]. During the fasting state, SIRT4 is inhibited

in liver. This induces gluconeogenesis from amino acids and fats and the inhibition of SIRT4 allows insulin secretion from β-cells. However, SIRT4 is activated and the reactions mentioned above are reversed in the fed state[50].

In the early stages of type 2 diabetes mellitus, insulin resistance is the dominant feature and as a result hyperinsulinemia occurs. Impaired glucose uptake and utilization follow this stage and hyperglycemia and hyperinsulinemia contribute to pancreatic β islet cell destruction in the following stages of diabetes[52]. SIRT1 induces gluconeogenesis and inhibits glycolysis in liver during fasting by deacetylating FOXO1 and PGC1α. One of the most important questions is what are the changes in gluconeogenesis and glycolysis in diabetes mellitus? Rodgers *et al*[53] showed that hepatic PGC-1α is upregulated and gluconeogenesis is increased which can further aggravate hyperglycemia in diabetic mice. Yechoor *et al*[54] demonstrated that SIRT3 mRNA is down-regulated in muscle insulin receptor knock-out mice. Hallows *et al*[55] showed that SIRT3 induces ketogenesis by activating acetylCo-A synthetase in mammalian cells. Hence, one might expect that SIRT3 may play an important role in the increased ketogenesis observed during diabetes mellitus.

SIRT1, -3 and -4 play an important role in the pathogenesis of hepatosteatosis which is commonly seen in diabetic patients[56]. When taken together, inhibition of SIRT1 and 3 and/or activation of SIRT4 might be attributed to this heightened risk of hepatosteatosis in the progression of diabetes mellitus.

Dong *et al*[57] reported that there was an association between the *SIRT5* and *SIRT6* gene variants with atherosclerosis. Several important relationships were found between gender and risk factors including smoking (for the associations with SIRT5 and UCP-4), hypertension (for the associations with SIRT3, SIRT5, and UCP-5), and diabetes (for the associations with SIRT5 and UCP-5). These results suggest that genetic variants in sirtuins may have an influence on the development of vascular aging phenotypes, independent of common risk factors.

NOVEL THERAPEUTIC AGENTS OF SIRT1 REGULATORS IN THE TREATMENT OF DIABETES MELLITUS

A plant polyphenol, resveratrol, was found to be the first drug to activate SIRT1[58]. Recent research demonstrated that the positive effects of resveratrol on glucose metabolism and insulin sensitivity were closely associated with AMPK subunit α activation of this agent rather than the stimulatory effect on SIRT1. Um *et al*[59] showed that resveratrol did not improve glucose tolerance and insulin sensitivity in AMPK α knock-out mice. On the other hand, Timmers *et al*[60] recently demonstrated the beneficial effects of resveratrol in obese patients in terms of lowering systolic blood pressure, serum lipid and glucose levels and inflammation parameters.

There are conflicting results about the effects of novel synthetic SIRT1 activators on glucose and lipid metabolism. SIRT1 activators might induce insulin secretion and sensitivity, reduce adipogenesis, but also induce gluconeogenesis in the liver which may worsen hyperglycemia in diabetes mellitus. Recently, Yamazaki *et al*[61] showed that treatment of mice with nonalcoholic fatty liver disease with a synthetic SIRT1 activator, SRT1720, might decrease the serum lipid levels, oxidative stress and inflammation. In addition, Feige *et al*[62] suggested that activation of SIRT1 *via* SRT1720 protected the organism from diet-induced obesity and insulin resistance by increasing oxidation of fatty acids in liver, adipose tissue and skeletal muscle.

Nicotinamide mononucleotide (NMN), a NAD^+ intermediate, is another molecule that has been demonstrated to have beneficial effects and improved glucose and lipid levels in aging-induced diabetes[63]. The role of NMN regarding diabetic nephropathy has also been studied. Recent studies showed that SIRT1 in proximal tubule cells protects against albuminuria in diabetes by maintaining NMN concentrations around glomeruli and controlling podocyte function[64,65]. In addition, SIRT1 was found to be closely associated with the survival of cells in an affected kidney by modulating their responses to various stress stimuli, SIRT1 also takes part in arterial blood pressure control, protects against cellular apoptosis in renal tubules by inducing catalase and triggers autophagy. Hence, activation of SIRT1 may become a novel target in the treatment of diabetic nephropathy[66].

Niacin (vitamin B_3), is also an important intermediate for the biosynthesis of NAD^+ that can used for the activation of SIRT1[67].

Metformin, a commonly used anti-diabetic drug, decreases insulin resistance and hyperglycemia by inhibiting gluconeogenesis and hepatic glucose output, and activation of free fatty acid oxidation in skeletal muscle[68]. Some of these beneficial effects of metformin were attributed to SIRT1 activation *via* the AMPK pathway[69].

Calorie restriction results in a desirable metabolic profile and improvement in mitochondrial function in humans by activating several genes including SIRT1[70]. In this regard, CR with increased physical activity should be encouraged especially in obese diabetic patients.

In contrast to the above-mentioned data regarding the beneficial effects of SIRT1 activation, Marampon *et al*[71] recently demonstrated that an angiotensin converting enzyme inhibitor, zofenoprilat, triggered SIRT1 downregulation *via* p38 activation. They concluded that zofenoprilat negatively controlled angiotensin I receptor protein expression through SIRT1 and this would be associated with improved cardiovascular morbidity and mortality especially in hypertensive and diabetic patients. Hence, further research is needed to clarify the exact role of the SIRT1-related pathways in the pathogenesis of diabetes and hypertension.

In summary, SIRT1 may represent a new therapeutic target for the prevention of insulin resistance, obesity, diabetes mellitus and its chronic complications[72]. However, to date, among the treatment options mentioned above, using metformin along with CR may the optimal choices

in obese type 2 diabetic patients.

CONCLUSION

Calorie restriction, oxidative stress, and various endogenous proteins might decrease nicotinamide and increase the NAD/NADH ratio that trigger sirtuins. In the fasting state, sirtuins inhibit insulin release in the pancreas and prevent β-cell degeneration, promote gluconeogenesis and insulin signaling, inhibit glycolysis and adipose tissue differentiation, and prevent ketogenesis, especially in diabetes mellitus. Activation of sirtuins may result in various beneficial metabolic effects which makes these proteins target new drugs, especially for the future treatment of metabolic disorders including diabetes and obesity. However, there are many missing pieces in the puzzle. Hence, further experimental and clinical studies are needed to highlight the exact roles of sirtuins in diabetes mellitus.

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MINIREVIEWS

Diabetes mellitus and hypothyroidism: Strange bedfellows or mutual companions?

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Abstract

Clinicians should be cognizant of the close relationship that exists between two of the most common endocrine disorders, primary hypothyroidism and diabetes mellitus. This applies to patients with both type 1 and type 2 diabetes mellitus (T1DM and T2DM respectively). However, the association is greater in T1DM, probably because of the shared autoimmune predisposition. In patients with T2DM, the relationship is somewhat weaker and the explanation less clear-cut. Factors such as dietary iodine deficiency, metformin-induced thyroid stimulating hormone suppression and poor glycemic control may all be implicated. Further translational research is required for greater clarification. Biochemical screening for abnormal thyroid function in individuals who have diabetes is warranted, particularly in females with T1DM, and therapy with L-thyroxine appropriately instituted if hypothyroidism is confirmed.

Key words: Type 1 diabetes; Type 2 diabetes; Primary hypothyroidism; Autoimmune disorders; Thyroid screening; Thyroid treatment

Core tip: Clinicians should be cognisant of the close relationship that exists between two of the commonest endocrine disorders, primary hypothyroidism and diabetes mellitus. This applies to both type 1 and type 2 diabetes. However the association is greater in type 1 diabetes, probably due to shared autoimmune predisposition. In type 2 diabetes, the connection is more complex. Biochemical screening for thyroid dysfunction in patients with diabetes is advised.

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INTRODUCTION

Two of the main clinical disorders encountered in endocrine clinics are diabetes mellitus and primary hypothyroidism. Diabetes can be divided into type 1 diabetes mellitus (T1DM), frequently the result of autoimmune islet-cell destruction, and T2DM, whose pathogenesis embraces both environmental and genetic components[1,2]. Primary hypothyroidism, on the other hand, usually follows autoimmune damage to thyroid tissue by circulating antibodies[3]. The concurrence of these two frequently encountered endocrine conditions in a particular patient has aroused much debate[4]. T1DM and primary hypothyroidism both share an autoimmune predisposition, while T2DM and hypothyroidism could be connected by the concurrence of two frequently occurring endocrine disorders.

The purpose of this review was to evaluate the evidence for an association of both T1DM and T2DM with hypothyroidism. The comparative frequencies of hypo-

Table 1 Prevalence of hypothyroidism in patients with type 1 diabetes *n* (%)

Gender	Number of subjects	Prevalence of hypothyroidism
Female	246	76 (30.9)[b]
Male	258	26 (10.1)
Total	504	102 (20.2)

[b]$P < 0.001$ *vs* males.

thyroidism in T1DM and T2DM were also assessed.

TYPE 1 DIABETES AND HYPOTHYROIDISM

Autoimmune thyroid disease is the commonest autoimmune disorder associated with T1DM[5]. This should not be surprising as T1DM and autoimmune thyroid disease share an autoimmune disposition, and recent studies have shown a shared genetic susceptibility to both conditions[6,7]. Regarding the shared genes involved in this immune predisposition, the *CTLA-4*, *HLA* class 11 and *FOXP3* genes have been implicated. Like T1DM, autoimmune thyroid disease is due to organ-specific autoimmunity. There is infiltration of the thyroid gland with T-lymphocytes and the formation of autoreactive antibodies, particularly against thyroglobulin and thyroid peroxidase (TPOAb). These antibodies are commonly found in patients with T1DM and may be present in up to 25% of patients with T1DM at the time of diagnosis of the diabetes[8]. The presence of thyroid antibodies is predictive of the later development of autoimmune thyroid dysfunction, usually hypothyroidism but also, less commonly, hyperthyroidism[9]. Umpierrez *et al*[10] reported that in patients with type 1 diabetes who had been followed for 18 years, those who were TPOAb-positive were much more likely to become hypothyroid than patients who showed negative antibodies at the outset.

Should hypothyroidism occur, even in a subclinical form, it may be associated with increased risk of hypoglycemia, by reduced hepatic glucose output and especially from impaired gluconeogenesis[11]. There may also be reduced linear growth in children and adolescents[12].

The prevalence of hypothyroidism in patients with T1DM has been estimated to be between 17% and 30%[5]. In our own recently published survey of T1DM at a private diabetes clinic in Johannesburg, South Africa[13], we found a 20.2% prevalence of hypothyroidism in 504 patients with established T1DM. Females showed a significantly higher prevalence than did males (30.9% *vs* 10.1%, $P < 0.001$) (Table 1). Our prevalence rate was slightly higher than that in a study by González *et al*[14], which involved smaller patient numbers. That report again emphasized that the presence of thyroperoxidase autoantibodies at T1DM onset was highly predictive for the development of subsequent thyroid dysfunction. In our survey, we also noted an increased prevalence of other organ specific autoimmune diseases such as Addison's disease, celiac disease and pernicious anemia, but at a much lower frequency.

TYPE 2 DIABETES AND HYPOTHYROIDISM

In T2DM, the association with hypothyroidism is more complex. It is unlikely to be a coincidence of two common endocrine disorders, since the prevalence of hypothyroidism is higher than in the general population. This has been demonstrated in a number of epidemiological studies including our own[15-18], with the prevalence of hypothyroidism varying between 11% and over 30% across different ethnic groups, as opposed to 4% reported in the general population[3-19]. The presence of undiagnosed hypothyroidism may increase cardiovascular risk by aggravating dyslipidemia, insulin resistance, obesity and vascular endothelial dysfunction[20,21]. Factors that could be implicated in this association are rather ill defined and may be complex. Insufficient iodine intake in the diet is one possibility, since a recent study highlighted reduced iodine consumption in 3 major American weight reducing programmes[22]. A report documenting a TSH-lowering effect of metformin in T2DM[23] may also be relevant, although the relationship between metformin and hypothyroidism is likely to be a complex one. Our study suggested that metformin usage might actually be protective against hypothyroidism in patients with T2DM or perhaps that suppressed thyroid-stimulating hormone caused by metformin may lead to physicians missing the diagnosis when thyroid-stimulating hormone measurement is the only screening method employed[16]. Additionally, poorly-controlled diabetes may induce alterations in thyroid function tests similar to that occurring in systemic illnesses i.e. lower levels of all thyroid hormone measurements[24]. Finally the possibility of alterations in the gut microflora being detected in both T2DM and thyroid dysfunction warrants attention. Further studies are clearly required to clarify the causal relationships between these two major endocrine disorders.

COMPARATIVE FREQUENCIES OF HYPOTHYROIDISM IN TYPE 1 AND TYPE 2 DIABETES

From our own large database of patients with diabetes in Johannesburg, we were able to establish that the overall frequency of diagnosed hypothyroidism in T1DM was almost double that seen in T2DM (Table 2). This applied to both female and male subjects. The closer association of hypothyroidism with T1DM probably reflects their well-established autoimmune predisposition and confirms the clinical observation that patients with one organ-specific autoimmune condition are at risk of developing other autoimmune diseases[25].

Table 2 Comparative prevalence of hypothyroidism in patients with type 1 and type 2 diabetes *n* (%)

Diabetic subgroup	Number of subjects	Prevalence of hypothyroidism
Type 1	504	102 (20.2%)[b]
Type 2	918	108 (11.8%)

[b]$P < 0.001$ *vs* type 2 diabetes.

RECOMMENDATIONS FOR THYROID SCREENING AND THERAPY

Hypothyroidism can be clinically silent or aspects of poor diabetes metabolic control may mask its clinical features. In view of the extremely high prevalence of hypothyroidism in those with T1DM, screening for thyroid disease should be done in a systematic fashion. Regular screening will unmask a substantial number of individuals with asymptomatic thyroid dysfunction. Current guidelines advise screening type 1 diabetic subjects at the time of diagnosis or initial contact[26,27].

Thereafter, it is recommended that the TSH is measured annually or two-yearly, but more frequently in antibody-positive patients or individuals who develop a goiter[28]. In the event of pregnancy, this becomes a necessity to prevent damage to fetal mental development secondary to undiagnosed maternal hypothyroidism[29].

For patients with T2DM, the recommendations for biochemical screening are less obvious and depend on factors such as sex, ethnic origin and age. Advice regarding routine testing is either vague[27] or firmly against routine yearly screening of type 2 diabetic patients[28]. Gopinath *et al*[30] reported no difference in the 5-year incidence of thyroid dysfunction in elderly patients with and without diabetes and another study by Chubb *et al*[31] also reported no development of frank hypothyroidism in female type 2 diabetes who manifested subclinical disease. This is in contrast to the data presented in this review, which highlights the increased prevalence of hypothyroidism in patients with T2DM. Selective periodic testing of patients with T2DM is probably warranted. Thyroid antibodies and serum thyroid stimulating hormone (TSH) levels are a useful means of identifying patients with diabetes who are at the greatest risk of thyroid dysfunction. Serum TSH concentrations in the upper range of normal appear to predict the development of future hypothyroidism. In one study involving subjects with both T1DM and T2DM, a TSH concentration above 1.53 mU/L predicted later hypothyroidism[32]. Therefore those with TSH concentrations in the upper normal range probably warrant more frequent, perhaps annual, re-testing. Regarding therapy in patients with diabetes, L-thyroxine should be instituted after confirmed biochemical diagnosis. Since patients with T2DM frequently have underlying ischemic heart disease, therapy in these patients should be started at low dosage (*e.g.*, 25 μg daily). This should be gradually increased over time, using the serum TSH level as a marker of adequate replacement. A serum TSH between 0.5 and 2.0 mU/L is generally considered the optimal target range to aim at[33].

CONCLUSION

Diabetes and hypothyroidism are indeed mutual companions based on the clinical studies that we have reviewed. This applies both to patients with T1DM and T2DM, although patients with T1DM are most predisposed. However, in both subtypes of diabetes, females are more vulnerable to develop hypothyroidism. Clinicians should be alerted to the close relationship that exists between these two common endocrine disorders and the importance of biochemical screening for hypothyroidism as indicated above. Appropriate thyroid replacement therapy can be introduced at an early opportunity, when required.

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MINIREVIEWS

Diabetes mellitus in Nigeria: The past, present and future

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Abstract

Diabetes mellitus (DM) is a diverse group of metabolic disorders that is often associated with a high disease burden in developing countries such as Nigeria. In the early nineties, not much was known about DM in Nigeria and traditionally, people related DM to "curses" or "hexes" and diagnosis was made based on blood or urinary tests for glucose. Currently, oral hypoglycaemic agents but not insulin are readily accessible and acceptable to persons with DM. The cost of diabetes care is borne in most instances by individuals and often payment is "out of pocket"-this being a sequel of a poorly functional national health insurance scheme. An insulin requiring individual on a minimum wage would spend 29% of his monthly income on insulin. Complementary and alternative medicines are widely used by persons with DM and form an integral component of DM care. Towards reducing the burden of DM in Nigeria, we suggest that there be concerted efforts by healthcare professionals and stakeholders in the health industry to put in place preventative measures, a better functioning health insurance scheme and a structured DM program.

Key words: Diabetes mellitus; Prevalence; Costs; Insulin; Burden

Core tip: This manuscript at best is a critical appraisal of earlier knowledge and data on diabetes mellitus (DM) in Nigeria. It also highlights the changes that have occurred in terms of prevalence and also in terms of diagnosis and management techniques. Challenges in provision of DM care and the roadmap for the future of DM care are documented in this manuscript.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disorder that is not only assuming pandemic proportions worldwide but also poised to affect the developing countries of the world much more than their developed counterparts. As far back as the beginning of the twentieth century, DM was described by Dr. Cook as being an uncommon disorder in the African. There is however, compelling data to show an increasing incidence and prevalence of DM in the continent[1]. The estimated prevalence of diabetes in Africa is 1% in rural areas, and ranges from 5% to 7% in urban sub-Saharan Africa[1].

Nigeria, with a population of 158 million people, is the most populous country in Africa and accounts for one sixth of Africa's population. Approximately 50% of Nigerians are urban dwellers and the country has a cultural diversity and 398 documented ethnic groups[2]. Health care delivery as in most developing countries of the world is at best sub-optimal and this may be respon-

sible for the dismal health indicator statistics such as reduced life expectancy at birth and increased maternal mortality. Health care provision in Nigeria is a concurrent responsibility of the three tiers of government with private providers of health care also playing a notable role in in health care delivery. Health insurance is still taking tottering steps despite having being inaugurated about two decades ago and healthcare payment is largely "out of pocket".

In this review, we attempt to document the present and past data on DM in Nigeria, and highlight the challenges of DM care. This article aims to appraise the present status of DM in Nigeria and the roadmap for the provision of DM care for the future.

Methods

We searched MEDLINE and reference lists of literature on diabetes in Nigeria from all available years and the keys words "diabetes", "prevalence" and "Nigeria" were used. For an extended search we introduced key words like complications. The combination of key words like "heart failure", "cardiovascular disease", "stroke", and "sexual dysfunction" nephropathy, and retinopathy. We also used search engines such as Google and Google scholar. The pattern of articles obtained included mainly retrospective and a few prospective studies and were largely hospital based with a few community based reports all drawn from urban and rural communities.

THE PAST

Studies that were conducted over the four decades from 1960 to 2000 showed generally low prevalence rates for diabetes in Nigeria[3-7]. Two studies[3,4] that were conducted in 1963 and 1971 reported prevalence of less than 1% for diabetes in Nigeria. The prevalence was still low at 0.8% to 2.8% in several studies[5-8] that were conducted from 1988 to 1998 with most patients having non-insulin dependent (type 2) diabetes. These studies[5-7] were limited to particular population groups in Nigeria except one[8] which was part of a national survey that assessed the prevalence of non-communicable diseases in the entire Nigerian population. In the past, diabetes was largely categorized as juvenile onset (insulin dependent) and maturity onset (non-insulin dependent) diabetes with juvenile onset diabetes being rarely reported in the Nigerian. The rarity of juvenile onset (type 1) diabetes is underscored by a study[9] that was done in 1990 where only 6% of 756 registered diabetes patients were aged 15 to 30 years at diagnosis. There used to be a class of diabetes referred to as malnutrition related diabetes, and this comprised of two subsets: fibrocalculous pancreatic diabetes and protein deficient diabetes[10]. Two Nigerian studies reported prevalence rates for Malnutrition related DM of 6%[10] and 8.6%[4]. Malnutrition related diabetes which was typically diagnosed in nutritionally deprived populations was however, removed as a separate class of diabetes in 1997 and rather considered as one of "other specific causes of diabetes"[11].

As far back as 1963 temporary diabetes had been described in adult Nigerians with the phenotypic characteristics of type 2 diabetes[12]. The term remittent DM was employed for the same phenomenon in 1978[13]. The more recent terminologies for this phenomenon where persons with phenotypic characteristics of type 2 diabetes present with unprovoked hyperglycaemic ketoacidosis as the initial manifestation of diabetes as expected with type 1 diabetes but subsequently run a course similar to Type 2 diabetes where they are insulin independent for several years has being described as Ketosis prone type 2 diabetes[14].

The earliest studies on the genetic contributions to the aetiology of DM in Nigeria found gene associations that are different from those reported in Caucasian populations[15,16]. While HLA-B8 is strongly associated with insulin dependent diabetes in Caucasians, the contrary was the case in Nigerians[15]. Another study[16] reported a low prevalence of DR4 in Nigerians with type 1 diabetes.

A study[17] that assessed patients' knowledge and self care practices of diabetes found that 78% of the Study population ascribed diabetes to poisoning and that about 70% of patients checked glycaemic control by tasting urine or passing urine on the ground and observing for ants.

Treatment of DM in Nigeria has always included the administration of insulin and oral hypoglycaemic agents in conjunction with dietary counselling and life style modification. Bovine and porcine insulin were the predominant forms of insulin used in the past. The animal insulins and particularly porcine insulin had the problems of immunogenicity which mitigated against their effectiveness[18]. Insulin treatment in the past was also complicated by the presence of various insulin concentrations and various sizes of insulin syringes namely the U40 for 40 units per milliliter vial and syringe and the U80 for the 80 units per milliliter vial as there was no proper regulation of the insulin market. There were often cases of patients getting discordant insulin vials and syringes leading to either hyperglycemia or hypoglycemia.

THE PRESENT

The current prevalence of DM in Nigeria is not known but guestimates may likely be in the region of 8%-10%. Of the four classes of DM, three types are frequently recognized in our setting and these are type 1 DM (T1DM), T2DM and gestational diabetes. Of the three types of DM, T2DM is the commonly documented form of DM and in most endocrine clinics, it accounts for about 90%-95% of all cases of DM. The prevalence of T1DM is not known but there are sketchy reports from various endocrine centres and documented prevalence rates which are all hospital based range from 0.1/1000 to 3.1/1000[19,20]. It is pertinent to note that in our setting, clinical criteria are often used to classify patients with DM into type 1 and T2DM. These criteria include a cut

off age of thirty years and insulin requirements or usage since diagnosis. For T2DM additional clinical criteria for diagnosis include history of usage of oral hypoglycaemic agents or usage of combination of insulin and the oral hypoglycaemic agents.

Gestational diabetes refers to any degree of glucose intolerance first detected in pregnancy. Patients diagnosed with diabetes in the first trimester of pregnancy are however more likely to have pre-gestational diabetes. One Nigerian study[21] found that gestational diabetes to occur in 2.98 per 1000 pregnancies, while another study[22], showed that the prevalence increased with maternal age; 3.3% in the age group of 15 to 24 years, 4.2% in those aged 25 to 34 years with a spike to 17.6% in the age group of 34 to 44 years and an average prevalence of 4.2%.

Gestational diabetes is usually first tested for in persons at risk between 24 and 28 wk gestational age. Gestational diabetes can be diagnosed using fasting plasma glucose, 75 gram oral glucose tolerance test (OGTT) or 100 g OGTT. Gestational diabetes is diagnosed based on the finding of fasting blood glucose ≥ 5.1 mmol/L-6.9 mmol/L (92-125 mg/dL) or plasma glucose 2 h post 75 g OGTT of ≥ 7.8 mmol/L[23]. Where 100 g OGTT is performed, gestational diabetes is diagnosed when at least 2 results of blood samples taken at fasting, 1, 2 or 3 h post OGTT meets the following threshold values; fasting plasma glucose ≥ 5.3 mmol/L, 1 h post OGTT ≥ 10 mmol/L, 2 h post OGTT ≥ 8.6 mmol/L and 3 h post OGTT ≥ 7.8 mmol/L[23].

The Diabetes Association of Nigeria recommends the performance of the 75 g OGTT in pregnant work with risk factors for gestational diabetes. These risk factors are a previous history of gestational diabetes, family history of type 2 diabetes, pre-pregnancy body mass index ≥ 25 kg/m^2, birth of baby > 4 kg, recurrent miscarriage, still birth, neonatal death, grand multiparity, polycystic ovarian syndrome, systemic hypertension and glycosuria in index pregnancy. Patients diagnosed with gestational diabetes during pregnancy will need to be re-assessed about 6-12 wk post-delivery using fasting plasma glucose and or plasma glucose at 2 h post 75 g OGTT interpreted using criteria applicable to non-pregnant adults[24].

For the diagnosis of DM the World Health Organization (WHO) 1999 criteria apply[25] and the commonly used test is the fasting plasma glucose which is more pragmatically poised in the diagnosis of DM than the oral glucose tolerance test that is not readily reproducible. The use of glycosylated haemoglobin test in the diagnosis of DM was recommended by the WHO in 2011 and a level of ≥ 6.5% (≥ 48 mmol/mol) was taken as a cut-off for diagnosing type 2 diabetes in non-pregnant adults[26]. Using HbA1c for diagnosis requires the International Federation of Clinical Chemistry standardised assays for its measurement to ensure the results produced using different assays are equivalent and reliable[27]. In Nigeria, glycated haemoglobin levels are more often than not determined by point-of-care tests which are not standardized for use in diagnosing diabetes.

Management of persons with DM is composed of non-pharmacological and pharmacological components. We routinely offer both components of care to persons with DM even though most centres tend to underemphasize the non-pharmacological aspect paying attention mainly to the dietary aspect.

A component of comprehensive DM care as recommended by the American Diabetes Association includes a yearly laboratory evaluation for lipid profile, liver function test, serum creatinine and calculated glomerular filtration rate, test for spot albumin excretion and thyroid stimulating hormone in persons with T1DM, dyslipidaemia and women over 50 years of age[24].

Dietary management is a key cornerstone modality in the attainment of good glycaemic control in DM. Dietary management of DM is targeted at improving the overall health by achieving and maintaining optimal nutritional status, attaining good glycaemic control and prevention of acute and long term complications of DM. There is no standardized diet for people with DM and the dietary requirements for people living with DM often are influenced by, socio economic status, religious beliefs and cultural beliefs. The current general recommendation is that carbohydrates should provide between 45%-65% of the daily caloric intake, fat should be 25%-35% of total daily calories and protein 15%-20% should be of total daily calories[28]. In Nigeria there is the erroneous beliefs amongst many people that DM results from eating carbohydrates hence the popular view that people with DM should either completely avoid carbohydrates or at best take minimal quantities. The resultant sequelae of these wrong notions include the intake of monotonous meals which are deemed "safe" for people with DM. One of such meals that are commonly prescribed by well-meaning non healthcare professionals and uninformed medical personnel include unripe plantain and beans. In a report by Abioye-Kuteyi *et al*[29] on dietary knowledge and practices in persons with T2DM, about half of the Study subjects ate a monotonous diet of mainly plantain and did not necessarily attain good glycaemic control.

These erroneous beliefs concerning dietary requirements in DM also affect the stance of patients when faced with the occurrence of iatrogenic hypoglycaemia. Some patients with DM have been noted to absolutely refuse simple sugars in the management of this life threatening acute complication of DM. There are varying Nigerian reports[29,30] that note that adherence to dietary advice is often poor amongst people with DM. Dietary management as an aspect of DM care is seen as the turf of the nutritionists and as a result, quite a number of physicians have a poor know how on dietary counselling. Exercise is known not only to impart glycaemic control positively but also to reduce the risk of developing cardiovascular disease in DM.

DM is a diverse group of metabolic disorders that is often associated with a high disease burden in developing countries such as Nigeria. In the early nineties, not much

Table 1 Drugs that are currently employed in the management of diabetes mellitus in Nigeria

Oral glucose lowering agents
Biguanides
Sulphonylureas
Alpha-Glucosidase inhibitors
DPP-4 inhibitors
Parenteral glucose lowering agents
Human insulin
NPH insulin
Insulatard
Premixed (30/70)
Insulin analogues
Insulin glargine
Insulin lispro
Premixed: Novomix, Humalog (25/75)

DPP-4: Dipeptidyl peptidase 4.

was known about DM in Nigeria and traditionally, people related DM to "curses" or "hexes" and diagnosis was made based on blood tests or urinary tests for glucose. Currently, oral hypoglycaemic agents but not insulin are readily accessible and acceptable to persons with DM. The cost of diabetes care is borne in most instances by individuals and often payment is "out of pocket"-this being a sequel of a poorly functional national health insurance scheme. An insulin requiring individual on a minimum wage would spend 29% of his monthly income on insulin. Complementary and alternative medicine are widely used by persons with DM and forms an integral component of DM care.

Towards reducing the burden of DM in Nigeria, we suggest that there be concerted efforts by healthcare professionals and stakeholders in the health industry to put in place preventative measures, a better functioning health insurance scheme and a structured DM program.

The American Diabetes Association recommends that individuals with T2DM perform at least 150 min of moderate-intensity aerobic exercise and/or at least 90 min of vigorous aero-bic exercise per week[27]. The erroneous impression amongst lay people that exercise should be performed with an intention to lose weight is all too pervasive in our practice. Exercise prescription is hardly done and when offered some physicians offer generic advice on exercise. In the Diabcare Study in Nigeria, only a third of persons with DM admitted to exercise adherence[31].

The importance of self-glucose monitoring is known to the majority of persons living with DM even though this knowledge does not necessarily translate into implementation. The practice of self-glucose monitoring in DM ranges from 3.4% amongst patients with DM in rural settings to 73% in urban settings[32-34]. Despite the limitation of urine testing, some patients still employ this technique for self-monitoring of glycaemic control. A Nigerian Report have noted that some patients with DM monitored glycaemia using urine tests with the aid of Clinitest tablets, urine dipsticks and in some rare instances, tasting the urine for sweetness[33]. In some centres in the more industrialized parts of the Nigeria the practice of urine testing for glucose is obsolete[34,35]. Beyond, financial constraints, psychosocial factors have been noted to largely influence glucose monitoring in our setting[35].

Pharmacological treatment of DM is composed of both insulin and oral glucose lowering drugs and in some instances complementary and alternative medicine. Effective usage of insulin in the management of glycaemia remains a challenge in developing countries like Nigeria and about a fifth of persons with T2DM are on insulin therapy solely or in combination with oral glucose agents[36].

Currently available human insulins are the short acting or meal time insulins, premixed insulin and the long acting insulins. The insulin analogues were introduced into the Nigerian market about three years ago and are still not readily accessible in terms of availability and affordability. Premixed analogues are the types of insulin analogues that are predominant and only one long acting analogue (glargine) is available in the country till date. Insulin administration devices such as the syringes and prefilled pens are readily available but insulin syringes are the dominant forms of devices in use. Unfortunately there is no uniformity or standardization of insulin syringes in use and this is because of parallel importation of drugs and an absence of gazetted policies on DM management. The barriers to insulin usage include patient factors such as needle phobias, fear of hypoglycaemia, weight gain and costs. Healthcare provider factors include inertia on commencing insulin and this may be presumably a result of ignorance on when to start insulin and sometimes misguided attempts to "empathise" with the patients. In a report by Ogbera *et al*[36], well over half of persons on insulin paid for their insulin themselves and the mean costs of procuring insulin per month was determined to be about 37 dollars per month. The Report also noted that persons on minimum wage spent 29% of their monthly salaries in the procurement of insulin.

Oral hypoglycaemic agents (OHAs) are readily available and commonly used OHAs are metformin, glimepiride and glibenclamide. Other available therapies, *viz*., thiazolidinediones, alpha glucosidase inhibitors and the dipeptidyl peptidase 4 inhibitors are prescribed mainly by endocrinologists. Although OHAs are clearly not indicated for use in persons with T1DM, there are few cases of persons with clinical features of T1DM being placed on OHAs by general practitioners. A summary of glucose lowering agents used in the management of DM in Nigeria is shown in Table 1.

Complementary and alternative medicine (CAM) usage is an important facet of management of DM and a Nigerian Report noted that 46% of persons with DM used CAM with biological based therapies being the prevalent forms of CAM utilized[37].

A commonly used CAM therapy for the DM and hypertension is vernonia amygdalina which in local parlance is known as "bitter leaf", the widely held belief is that the bitter taste of this therapy counteracts the "sweetness" in the blood. This view although appears simplistic, may

have some scientific basis as Nigerian researchers have reported a lowering of blood glucose in diabetic rats and this lowering of glucose was comparable to that recorded in diabetic rats who had oral glucose lowering agents administered to them[38,39].

The burden of DM is attributable to complications which may be acute or chronic. Hyperglycaemic emergencies remain a major cause of concern in Nigerians with DM, accounting for 40% of all DM admissions with documented determinants of fatal outcomes being DM foot ulcers, hypokalaemia and sepsis[40,41]. Of all DM admissions hyperglycaemic emergencies are listed as one of three complications of DM associated with high case fatality rates[42]. Foot ulceration is one complication of DM that is widely reported on with a prevalence rate of about 9.5%[43]. Foot ulceration is reported to occur in 25%[44] of all new cases of DM and associated with an in-hospital mortality rate of 43%[45]. A major risk factor for DM foot ulceration is neuropathy (and this is eminently preventable. However in terms of treating the diabetic foot, not much progress has been made but preventative strategies with a focus on patient education have greatly improved.

Diabetic nephropathy is assuming an increasing role as a cause of chronic kidney disease in Nigeria and it is one of the leading cause of chronic kidney disease in patients starting renal replacement therapy. DM nephropathy is associated with increased cardiovascular risk. Cardiovascular complications of DM such as Stroke, and peripheral disease have been reported in 11%[46] and 37%[47] of persons with DM respectively in hospital settings in Nigeria. DM has also been noted to account for 2.1% of cases of heart failure[48]. Conventional cardiovascular risk factors such as hypertension, metabolic syndrome and dyslipidaemia are now routinely screened for in persons with DM and the use of statins and antiplatelet drugs are on the increase more than ever before in DM clinics. Novel cardiovascular risk factors such as elevated C reactive protein, and lipoprotein are not screened for routinely and remain issues of research concerns.

Diabetic retinopathy is a leading cause of blindness in people with DM and accounts for 16.2% to 42.1%[49,50] of retinal diseases. Unfortunately investigative techniques such as fluorescein angiography, and interventions such as laser treatment are not readily available for the detection and management of some of these eye complications of DM.

Erectile dysfunction is a prominent clinical feature of hypogonadism and usually associated with low testosterone levels. A third of all males with DM present with the testicular deficiency syndrome but less than half of these patients discuss this problem with their care givers[51]. A lot of unlicensed therapies are in the Nigeria market for treating erectile dysfunction but medical therapies available include the PDE 5 inhibitors, testosterone injections and the vacuum device which was introduced this year-2014-but is yet to gain wide acceptance. Sexual dysfunction in women with DM is an understudied aspect of DM complications and often there are no interventions offered in our locale. Whilst the occurrence of sexual dysfunction in women with DM is comparable to that of women without DM, psychological morbidity appears to be a contributory factor in women with DM[52].

Managing diabetes involves stakeholders of which national bodies on DM play a vital role. There are two umbrella bodies that serve the interest of DM in Nigeria and these are Diabetes association of Nigeria and the Endocrine and metabolic society of Nigeria. The afore stated bodies are charged with articulating guidelines on DM and also collaborating with policy makers and non-governmental bodies in order to reduce the burden of DM. At present, there is a National Guideline document on DM and a Lagos State Guideline-sponsored by Structured Healthcare Initiatives, an non governmental organization run by the primary author. The importance of having a clinical practice guideline document on DM cannot be overemphasized. A guideline document creates opportunity for assessment and standardisation of care, raising awareness on DM and empowering healthcare professionals at all levels of healthcare delivery at all locations (rural as well as urban areas) to detect and manage DM.

THE FUTURE

The keys issues with regards to diabetes in the future relate to the increasing population of Nigerians, increasing life expectancy of Nigerians, projected increase in the incidence and prevalence of diabetes, low per capita income of most Nigerians, poorly developed health care infrastructure and the current situation where the predominant means of procuring health services is "out of pocket" payment.

The aforementioned factors will result in increased numbers of persons with the complications of diabetes particularly against the backdrop of constrained health budget by various tiers of governments. Indeed the budgetary allocation to health for the 2014 fiscal year by the federal government of Nigeria at 6% remains less than 15% recommended by the WHO[53]. This is ironic as Nigeria was one of the African countries which participated in the 2001 Abuja[53]. There is need for government to increase the budgetary allocation for health as recommended by the WHO.

The prevention and improved management of diabetes will require cooperation between the government and the health sector. There is need for preventive programs such as enlightenment campaigns on the risk factors of diabetes. Government at all levels will need to improve health care funding.

The Health insurance scheme in Nigeria is poorly developed and currently, the majority of health insurance facilities do not provide coverage that allows for provision of optimum standard of care for persons living with DM. Out of pocket expenditure remains the major means of funding health care for the vast majority of Ni-

gerians now and in the foreseeable future.

The use of HbA1c for the diagnosis of diabetes remains limited by high cost. In one medical facility[54], it cost the equivalent of 19 USD to perform an HbA1c test. The relatively high prevalence of the sickle cell gene in Nigeria may impact on the assay for HbA1c.

Although several new agents have emerged for the treatment of diabetes such as insulin analogues, glucagon like peptide 1 analogues, amylinomimetics, inhaled insulin and insulin pumps, the country is probably better served by the regular availability of a few cheap diabetes medications with well-established safety profiles such as metformin, glibenclamide and gliclazide. Although lactic acidosis is a stated complication of metformin, the reality is that it is exceedingly rare even in patients with significant renal impairment and it has shown proven safety profile over decades of use.

There is the need for collaboration between healthcare providers, the pharmaceutical industries, policy makers and National agency for food and drug administration and control to ensure adequate regulation of the importation, local manufacture and use of anti-diabetic medications in Nigeria. Whilst the provision of continuous blood glucose monitoring systems are expensive for our economy, the use of standardized glucometers and test strips particularly for persons on multiple insulin injections needs to be encouraged. Some other areas of unmet needs include the availability of DM educators and podiatry specialists.

CONCLUSION

The status of provision of DM care has greatly improved in Nigeria but areas of concerns remain and some of these include financing and suboptimal patient education. Concerted effort should be put in plac by healthcare professionals and all stakeholders in ensuring that optimal care for persons with DM is attainable in Nigeria.

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MINIREVIEWS

Possible contribution of (pro)renin receptor to development of gestational diabetes mellitus

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Abstract

(Pro)renin receptor [(P)RR], a receptor for renin and prorenin, was first cloned in 2002. Since then, the pathophysiological roles of (P)RR have been growing concerns. (P)RR binds renin and prorenin, with two important consequences, nonproteolytic activation of prorenin, leading to the tissue renin-angiotensin system activation and the intracellular signalings. It is now also known to play an important role as vacuolar H^+-ATPase associated protein, involving in Wnt signaling, main component of embryonic development. Extracellular domain of full-length (P)RR is cleaved in golgi-complex forming soluble (P)RR [s(P)RR]. The s(P)RR is now possible to be measured in human blood and urine. It is now measured in different pathophysiological states, and recent study showed that elevated plasma s(P)RR levels in the early stage of pregnancies are associated with higher incidence of gestational diabetes mellitus later in the pregnancies. Plasma s(P)RR levels of neonates are known to be higher than that of adults. It was also shown that, increased s(P)RR concentrations in cord blood, associated with a lower small for gestational age birth likelihood. These data suggests the involvement of (P)RR in embryo's growth. In this review article, we attempt to figure out the possible pathophysiological roles of the (P)RR in maternal glucose intolerance and embryo's growth, through reviewing previous studies.

Key words: (Pro)renin receptor; Gestational diabetes mellitus; Embryonic growth; Renin-angiotensin system; Vacuolar H^+-ATPase; Wnt signaling

Core tip: Prorenin receptor [(P)RR] binds (pro)renin, and leads to the activation of tissue renin-angiotensin system and intracellular signalings. It also plays an important role as vacuolar H^+-ATPase associated protein, involving in Wnt signaling. Elevated plasma soluble (P)RR [s(P)RR] levels in the early stage of pregnancies are associated with higher incidence of gestational diabetes mellitus (GDM) during the third trimester. Also, elevated s(P)RR levels in cord blood, associated with a lower small for gestational age birth likelihood, suggesting the involvement of (P)RR in embryo's growth. Here we attempt to elucidate the possible pathophysiological roles of the (P)RR in GDM.

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INTRODUCTION

(Pro)renin receptor [(P)RR], a receptor for (pro)renin, was first identified in 2002[1]. The C-terminal domain of this receptor had been previously described as ATP6AP2 protein, which associated with a vacuolar H^+-ATPase (V-ATPase)[2], a proton pump essential for acidification of

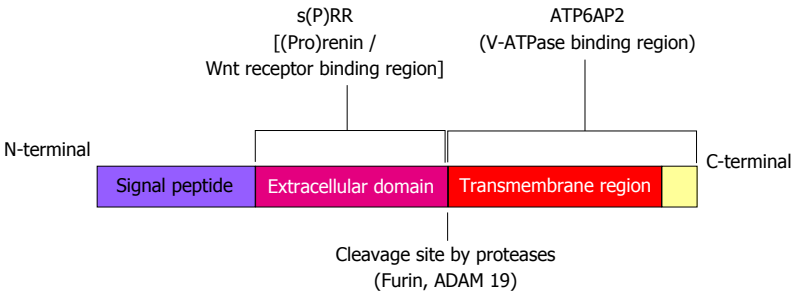

Figure 1 Structure of (pro)renin receptor. s(P)RR: Soluble (pro)renin receptor; V-ATPase: Vacuolar H^+-ATPase; ADAM 19: A disintegrin and metalloproteinase 19.

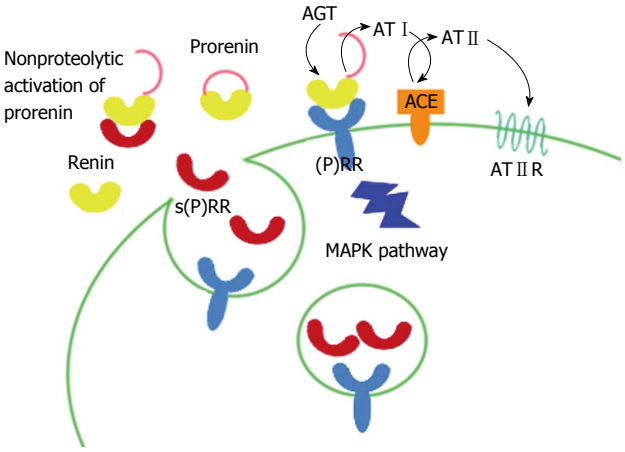

Figure 2 (Pro)renin receptor and (pro)renin. (P)RR: (Pro)renin receptor; s(P)RR: Soluble (P)RR; AGT: Angiotensinogen; AT Ⅰ: Angiotensin Ⅰ; AT Ⅱ: Angiotensin Ⅱ; ACE: Angiotensin converting enzyme; AT Ⅱ R: AT Ⅱ receptor; MAPK: Mitogen-activated protein kinase.

intracellular compartments. (P)RR consists of 350-amino acid with a single transmembrane domain and is known to exist in different molecular forms. Some exist as a full-length integral transmembrane protein, some as soluble (P)RR [s(P)RR] composed of extracellular domain, and other as truncated form composed of the transmembrane and cytoplasmic domains[3] (Figure 1).

When prorenin binds to (P)RR, a conformational change occurs in the prorenin molecule and gains full enzymatic activity without passing through proteolytic cleavage to renin[4]. Of different molecular forms of (P)RR, full-length and s(P)RR have a capacity of binding renin and prorenin. Thus, prorenin which is bound to either forms of (P)RR activates the tissue renin-angiotensin system (RAS) and for s(P)RR-bound prorenin, may also activate the circulating RAS. Also, when renin/prorenin binds to (P)RR, intracellular signaling pathways are triggered. *In vitro* experiments showed that the cell signalings are caused by both renin and prorenin in a manner independent of angiotensin[5-12] (Figure 2). Full-length and truncated (P)RR are capable of binding V-ATPase and are essential for V-ATPase assembly and function[13]. Extracellular domain of (P)RR binds Wnt receptor and serves as an adaptor for Wnt receptor and V-ATPase, and is now known to play important role in Wnt signaling, a key component of embryonic development[14-16].

Full-length (P)RR is known to be cleaved in the secretory pathway by proteases such as furin[3] and a disintegrin and metalloproteinase 19[17] to release s(P)RR into the circulation. Of the three different molecular forms, s(P)RR is the only molecule which is possible to be measured in human blood and urine samples. We have developed an s(P)RR enzyme-linked immunosorbent assay kit which allows quantification of s(P)RR in clinical settings[18]. The s(P)RR is now being measured in different pathological states. Recent study showed that increased plasma s(P)RR levels in pregnant women during the first trimester may predict the development of gestational diabetes mellitus (GDM) during the third trimester[19]. Plasma s(P)RR concentrations of neonates are higher than that of adults and the association between cord blood s(P)RR levels and small for gestational age (SGA) birth was shown[20], suggesting the involvement of (P)RR in embryo's growth.

In this review article, we make an attempt to figure out the possible pathophysiological roles of the (P)RR in pathogenesis of GDM and on embryo's growth.

(P)RR AND GLUCOSE INTORELANCE

Some data had shown the involvement of (P)RR on the pathogenesis of diabetes through angiotensin Ⅱ (Ang Ⅱ) production. The activation of prorenin, without undergoing cleavage to renin was observed and Ang Ⅱ contents increased in skeletal muscle tissues of fructose-induced rat models of insulin resistance[21]. Treatment with handle region peptide, inhibitory tool against prorenin binding (P)RR, markedly improved glucose tolerance, and this was associated with inhibition of nonproteolytic activation of prorenin by (P)RR and inhibition of increase in Ang Ⅱ contents. Insulin resistance observed in obese Otsuka Long-Evans Tokushima Fatty rats was also associated with nonproteolytic activation of prorenin and increase in Ang Ⅱ contents in the skeletal muscle and adipose tissues[22]. It has also been known that tissue RAS also exists in human pancreas and that it may directly affect β-cell function[23]. These findings indicate that (P)RR-bound prorenin may participate in the development of insulin resistance and β-cell function through tissue RAS activation.

Binding of (pro)renin to (P)RR also mediates Ang Ⅱ-independent signaling cascades. In vitro experiments

using the cells expressing the (P)RR showed the cell signaling caused by (pro)renin in an AngⅡ-independent manner. In the presence of angiotensin receptor antagonists, angiotensin converting enzyme inhibitors and/or renin inhibitors, the administration of prorenin/renin induced the activation of mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2, leading to upregulation of transforming growth factor β1, independent of AngⅡ generation[6,10,11]. (P)RR also activates the MAPK p38 and subsequent phosphorylation of heat shock protein[5,9], and the phosphatidylinositol-3 kinase-p85 pathway[24]. Since activation of MAPK and transforming growth factor-β1-dependent pathways induced by insulin are known to contribute to the pathogenesis of insulin resistance[25,26] and MAPK p38 cascade is considered to regulate β-cell function[27-29], (P)RR-induced activation of these intracellular pathways may also contribute to the pathogenesis of glucose intolerance.

(P)RR also plays important role as V-ATPase associated protein[13]. It has been reported that a3 isoform of V-ATPase regulates the exocytosis of insulin from pancreatic β-cells[30]. It has been also shown that V-ATPase is involved in insulin-stimulated glucose transport in 3T3-F442A adipocytes[31]. From these data, we may can hypothesize that (P)RR contributes to development of diabetes also through V-ATPase-linked functions.

MATERNAL (P)RR

Human RAS physiologically undergoes drastic changes during pregnancy. Since ovary and maternal decidua produces renin, early increase in plasma renin activity is seen during pregnancy. Circulating estrogen released from the growing placenta increases angiotensinogen synthesis by the liver, leading to increase in serum AngⅡ and aldosterone levels. Previous study has demonstrated that fasting blood glucose (FBG) in pregnant women is inversely correlated with the plasma renin activity, whereas plasma aldosterone concentration showed a significant positive correlation with FBG during pregnancy. Moreover, PAC is significantly higher in pregnant women with GDM as compared to those with normal glucose tolerance during pregnancy[32]. These data support an idea that the RAS during pregnancy is involved in the pathogenesis of GDM.

Plasma prorenin/renin ratio differs in each pathophysiological state. In the plasma, prorenin levels mark approximately 10-fold higher than renin levels in normal physiological condition[33]. In the diabetic patients and in pregnant women, plasma prorenin levels increase up to 50 to 100-fold higher than that of renin[34]. Particularly, plasma prorenin concentrations can be used as an early predictor of microvascular complications in the diabetic patients[35]. High levels of prorenin are also observed in infants. In these states in which plasma prorenin/renin ratio increases, (P)RR may play the main role in their pathophysiology.

(P)RR is abundantly expressed in placenta[1]. As mentioned above, higher levels of plasma s(P)RR in an early stage of pregnancy were significantly associated with a higher possibility of developing GDM in a later stage in pregnacy[19]. Women in the highest plasma s(P)RR level quartile were 2.90-fold more likely to develop GDM than women in the lowest quartile. This data also supports the theory that (P)RR may be involved in the pathogenesis of GDM.

FETAL (P)RR

S(P)RR levels in umbilical cord blood were significantly higher than that of normal adult[18]. In addition, high plasma s(P)RR level in cord blood is associated with a lower SGA birth likelihood[20]. Developmental studies in Xenopus and Drosophila have revealed an essential role of (P)RR to promote the canonical and non-canonical Wnt signaling pathways[16]. Wnt proteins form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis. Now that it is indicated that (P)RR plays key role in Wnt signaling, these data indicate that (P)RR may be essential for embryo's growth.

(P)RR POSSIBLY CAUSES GDM AS A RESULT OF STIMULATING AN EMBRYO'S GROWTH

Fetuses of mothers who have diabetes are more likely to be large for gestational age (LGA) than fetuses of non-diabetic women. From the data that high s(P)RR level in cord blood associates with a lower SGA birth likelihood[20], it can be speculated that plasma s(P)RR levels are also high in LGA fetuses. If the inappropriate growth stimulation of embryo precede the onset of maternal glucose intolerance, fetal s(P)RR may be a factor which triggers the onset of GDM. As full-length (P)RR does, s(P)RR also activates prorenin[36], thereby leading to the activation of RAS, resulting in development of GDM. However, there are some limitations to this hypothesis (Figure 3).

First, the mechanism of placental transfer of s(P)RR is unclear. It has been known that molecules larger than 1000 molecular weight is incapable of passing from fetal circulation to maternal circulation[37]. The s(P)RR may be too large to pass through placenta, since its molecular weight is 28000[38]. However, upstream factors which regulates the expression of (P)RR may pass through placenta from fetus, leading to the augmentation of (P)RR also in maternal tissues.

Second, it is now considered, regarding mechanism of LGA birth in GDM, that maternal glucose passes through placenta and induces fetal hyperglycemia leading to increase in plasma insulin levels[39]. This theory conflicts with our hypothesis that stimulation of embryo's growth precedes the development of GDM. However, increase in fetal (P)RR expression, as a result of hyperinsulinemia, may affect maternal pathological condition, creating a vicious cycle and at least in part explain the

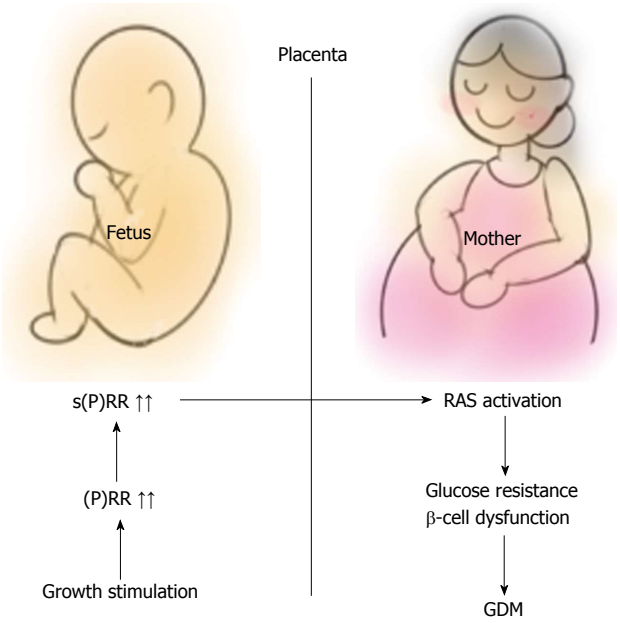

Figure 3 (Pro)renin receptor in the pathogenesis of gestational diabetes mellitus. (P)RR: (Pro)renin receptor; s(P)RR: Soluble (P)RR; RAS: Renin-angiotensin system; GDM: Gestational diabetes mellitus.

pathogenesis of GDM.

In conclusion, contribution of (P)RR to the pathogenesis of glucose intolerance has been speculated from previous studies. Although there is a lack of direct evidence, we highlighted the possibility of (P)RR-mediated fetal-maternal interaction as a pathogenesis of GDM. Measurement of maternal and cord blood s(P)RR levels in GDM patients at delivery will be needed to consolidate the theory. Also, time-course analysis of maternal and fetal s(P)RR in animal GDM model may provide evidences which may support pathogenetic role of (P)RR-mediated fetal-maternal interaction. Further investigations are needed, but this novel hypothesis may lead us to new diagnostic and therapeutic strategies for GDM.

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Nonalcoholic steatohepatitis and insulin resistance in children

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Abstract

Various pathological conditions can cause fatty liver in children. Nonalcoholic steatohepatitis (NASH) in children has been known since 1983. However, NASH diagnosed in childhood does not have a favorable outcome. The pathological characteristics of NASH are significantly different between children and adults. Nonalcoholic fatty liver disease (NAFLD)/NASH is accompanied by insulin resistance, which plays a pivotal role in its pathophysiology in both children and adults. In NASH, a "two-hit" model involving triglyceride accumulation (first hit) and liver damage (second hit) has been accepted. Insulin resistance was found to correlate with changes in fat levels; however, it did not correlate with fibrosis or NAFLD activity score in children. Therefore, insulin resistance may be important in the first hit. Because there is obvious familial clustering in NASH, genetic predisposition as well as environmental factors including diet might be the second hit of NAFLD/NASH.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Insulin resistance; Homeostasis model assessment as an index of insulin resistance; Obesity

Core tip: The pathological characteristics of nonalcoholic steatohepatitis (NASH) are significantly different between children and adults. Nonalcoholic fatty liver disease is accompanied by insulin resistance, which plays a pivotal role in its pathophysiology in both adults and children. In NASH, a "two-hit" model involving triglyceride accumulation (first hit) and liver damage (second hit) has been accepted. Insulin resistance was found to correlate with changes in fat levels; however, it did not correlate with fibrosis in children. Insulin resistance may be important in the first hit. Genetic predisposition as well as environmental factors might be the second hit in children.

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INTRODUCTION

Fatty liver disease (fatty liver) is a general term for diseases caused by an accumulation of triglyceride (TG) in liver cells. Various pathological conditions such as Turner syndrome, abnormal mitochondrial and fatty acid metabolism, nephrotic syndrome, Down syndrome, and hormonal therapy can cause fatty liver in children. In adults, nonalcoholic fatty liver disease (NAFLD) is defined by fatty liver without obvious causes such as autoimmune hepatitis, viral hepatitis, or drinking history. Histologically, NAFLD is divided into 2 categories: that without (simple steatosis) and that with fibrosis, necrosis, and inflammation [nonalcoholic steatohepatitis (NASH)]. NASH is regarded as a severe form of NAFLD. According to

a population-based study, 4.8% of adults with NAFLD have been reported to develop liver cirrhosis within a mean observation period of 7.6 years[1]. NASH/NAFLD in childhood has been known since 1983[2]. In this review, we introduce the recent findings of pediatric NASH and insulin resistance.

ETIOLOGY

In Japan, 10% of the general population is estimated to have NAFLD, and 1% to have NASH. In adults with obesity and type 2 diabetes insipidus, the rates are higher[3]. A life-table analysis showed a reduction of life expectancy of up to 7 years in adults with obesity[4]. In children, the prevalence of NAFLD/NASH is estimated to be as high as 2.6%-9.6% in the United States and Asian countries, despite significant differences in race and ethnicity[5-7]. Insulin resistance is often accompanied by NAFLD/NASH, and plays a pivotal role in its pathophysiology[8,9]. The prevalence of insulin resistance in obese children foreshadows a worrisome trend for type 2 diabetes. It is estimated that 170 million children under 18 years worldwide are overweight or obese, which is more than 20% of all children in many countries[10]. According to the SERCH for Diabetes in Youth study, more than 20000 individuals below 20 years of age had type 2 diabetes[11]. According to the follow-up study by Feldstein *et al*[12], 4 out of 66 children with NAFLD developed type 2 diabetes 4-11 years after diagnosis. Moreover, during a 20-year follow-up study, 2 children died and 2 underwent liver transplantation for cirrhosis[12].

CLINICAL DIAGNOSIS

There are no specific symptoms associated with NAFLD and NASH in children. However, there is strong fatigability. Furthermore, obesity, sleep apnea, hypertension, hyperinsulinemia, and acanthosis nigricans are often observed. Visceral obesity is a risk factor. Obesity (body mass index of greater than + 2SD) or an increase in weight of 10% or more per year is likely to be present.

Diagnosis of NAFLD and NASH by conventional blood biochemical examination is difficult. Liver biopsy is required for a definitive diagnosis of NAFLD.

For diagnosis, children should be screened for the presence of HBs antigens, HCV antibodies, anti-mitochondrial antibodies, anti-nuclear antibodies, ceruloplasmin, α-antitrypsin, transferrin, *etc*. Approximately 20% of adults with NASH showed positivity for antinuclear antibodies (greater than 160 X)[13]. Similar findings that 7 out of 14 children with NAFLD were positive for antinuclear antibodies or anti-smooth muscle antibodies have been reported by others[14].

In NAFLD, the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are usually mildly increased (2-4 times), and the level of ALT is higher than AST[15]. In NAFLD, levels of alkaline phosphatase and γ-glutamyl transferase are occasionally mildly increased. Levels of ALT and AST are higher in NASH than in NAFLD. Patients with cirrhosis show ALT/AST ratios of less than 1.

To differentiate between simple fatty liver and NASH, information on high-sensitivity C-reactive protein levels and insulin resistance [homeostasis model assessment as an index of insulin resistance (HOMA-R) (fasting blood glucose × immunoreactive insulin/405), adipocytokines [tumor necrosis factor (TNF)-α, adiponectin, and leptin], and oxidative stress markers] can be useful[16]. Other markers for NASH such as high levels of serum iron and ferritin, low platelet count, and KICG (same indocyanine green elimination rate constant) and fibrosis markers (hyaluronic acid, type Ⅳ collagen, and procollagen Ⅲ polypeptide) are also used. The NAFIC (NASH, ferritin, insulin, type Ⅳ collagen 7S) score for adults, pediatric NAFLD fibrosis index for children, and enhanced liver fibrosis test are useful to diagnose fibrosis[17].

Matteoni *et al*[18] classified NAFLD into 4 types from pathological findings. Type 1 is simple fatty liver (only fatty liver), type 2 demonstrates steatohepatitis (fatty liver and lobular inflammation), type 3 demonstrates steatonecrosis and ballooning and swelling of hepatocytes, and type 4 demonstrates steatonecrosis and Mallory bodies (liver cell ballooning degeneration) or fibrosis. He also reported the prognosis of each type upon long-term follow-up. Progression to liver cirrhosis or liver-related death were observed in patients with type 3 or 4 NAFLD. There were no cases that progressed to cirrhosis from types 1 and 2. Therefore, types 3 and 4 NAFLD are defined as NASH pathologically[18]. The grading system of necrosis and inflammation and the staging system of fibrosis that was defined by Brunt *et al*[19] are commonly used. On the other hand, NAFLD/NASH demonstrate different characteristics in adults and in children (Table 1)[20]. Figure 1 shows representative liver pathology of adult type and pediatric type NASH.

NAFLD/NASH in most children mainly have the characteristics of fatty changes, inflammation and fibrosis of the portal area, and absence of perisinusoidal fibrosis and hepatocyte ballooning. Patients with strong fibrosis are classified as having type 2 NAFLD/NASH. Schwimmer *et al*[21] classified pediatric NAFLD into 2 types. According to Brunt's pathological classification, the grading of necrosis and inflammation will be very low and staging of fibrosis will be very high in many children. NASH in children requires careful long-term observation.

BASIC PATHOLOGY

The phenotype of NAFLD is metabolic syndrome of the liver, which in general is accompanied by obesity, diabetes mellitus, hyperinsulinemia, and hyperlipidemia. In the onset and progression of insulin resistance and associated obesity, increased free fatty acid (FFA) levels and abnormal adipocytokine secretion are important factors. In NASH, a "two-hit" model involving TG accumulation (first hit) and liver damage (second hit) has been proposed[22].

Deposition of TG in liver cells is determined by the

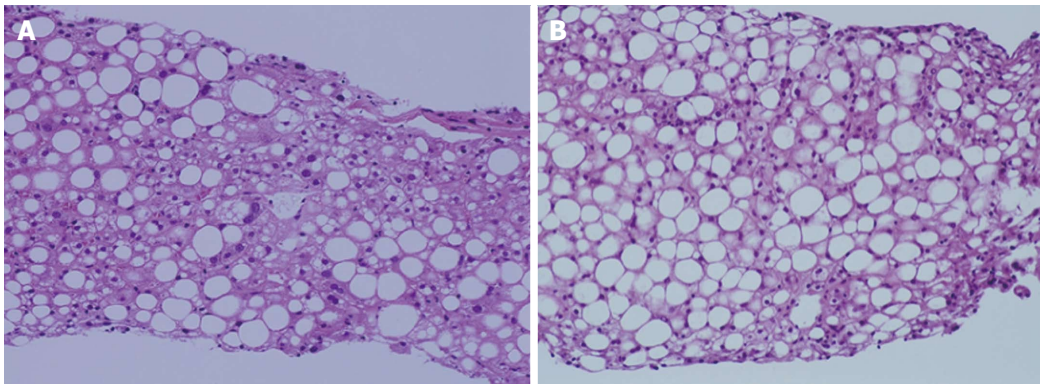

Figure 1 Representative photographs of liver sections of nonalcoholic steatohepatitis/nonalcoholic fatty liver disease patients. A: Pediatric type (type 1) showing severe fibrosis; B: Adult type (type 2) showing mild fibrosis and hepatocyte ballooning.

Table 1 Differences in characteristics of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis between adults and children

	Pediatric-type NASH	Adult-type NASH
Classification by Schwimmer *et al*[21]	Type 2	Type 1
Incidence	Frequent	Rare
Steatosis	Strong Starting in periportal zone (acinar zone 1)	Weak Starting in perivenular zone (acinar zone 3)
Inflammatory cell infiltration	Portal area	Centrolobular area
Hepatocyte ballooning	None	Prevalent
Fibrosis	None or only in periportal zone (acinar zone 1)	Prevalent in perisinusoidal or perivenular zone (acinar zone 3)
Liver cirrhosis	Present	Present
Epidemiology	More common in overweight, colored race (Hispanic: 73%; Asian: 12%), boys > girls	Hispanic: 41%, White, non-Hispanic: 53%, girls > boys
Ratio in pediatric NAFLD (overlap 16%) by Schwimmer *et al*[21]	51%	17%
Ratio in pediatric NAFLD (overlap 50%) by Takahashi *et al*[20]	21%	Not reported

NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease.

balance of TG-increasing factors (synthesis and influx of TG in liver cells) and TG-decreasing factors (efflux and consumption of TG in liver cells). TG is a molecule composed of 3 fatty acids esterified to a glycerol. Four mechanisms are assumed to affect the level of TGs in the liver cells. The first is increased uptake of FFA from food (15% of TGs in liver) and fatty tissue that supplies the FFA pool in the blood. TG from food is hydrolyzed to FFA by lipoprotein lipase. Non-hydrolyzed TG is supplied to liver cells directly. FFA from fatty tissue in the blood is absorbed by liver cells. Secretion of FFA from adipose tissue is increased when there is insulin resistance. The second is increased FFA synthesis in liver cells (*de novo* synthesis) or reduction of the suppression of FFA synthesis. Fatty acids derived from adipose tissue account for the majority (60%) of hepatic TG accumulation in NAFLD[23]. Nutrients such as carbohydrates, proteins, and lipids are converted to acetyl-CoA and serve as substrates for fatty acid synthesis. The third mechanism is decreased catabolism of FFA in liver cells (consumption by peroxisomes and mitochondrial β-oxidation). The fourth mechanism is decreased release of TG from liver cells (very-low-density lipoprotein is released into the blood by microsomal triglyceride protein)[24]. In children, total parenteral nutrition management, steroid administration, and fatty acid metabolism disorders are representative causes[25]. Oxidative stress, endotoxins, adipocytokines (TNF-α, adiponectin, and leptin) are considered as hepatocyte-damaging factors of the second hit. Hypoxia caused by sleep apnea also has a negative effect.

INSULIN RESISTANCE IN CHILDREN WITH NASH

The effects of steatohepatitis on insulin resistance in children have been elucidated recently. Cali *et al*[26] reported that in children with NASH, there was a significant decrease in insulin sensitivity and impairment in beta-cell function, as indicated by the fall in the disposition index paralleling the severity of hepatic steatosis[26]. Other reports also indicated that the deleterious effects of fat accumulation in the liver affect insulin sensitivity at a multi-organ level[11,27,28]. Consequently, insulin secretion becomes insufficient to maintain glucose levels and some obese children develop beta-cell impairment in the long run. In obese children, beta-cell function has

Table 2 Reports in the literature regarding insulin resistance in pediatric nonalcoholic steatohepatitis /nonalcoholic fatty liver disease

Ref.	Study population and sample size	Age (yr)	Method of diagnosis	Insulin resistance
Santoro *et al*[32]	229 obese children, including 12 cases of liver biopsy-proven NASH	12.8 ± 2.9	MRI and liver biopsy	No significant correlation between MRI-measured steatosis and whole body insulin sensitivity index
Fitzpatrick *et al*[33]	40 liver biopsy-proven NAFLD	10-16	Liver biopsy	68% showed insulin resistance. HOMA-R values did not correlate with NAS
Nobili *et al*[34]	30 NAFLD patients (11:19; without: with steatohepatitis)	8-14	Liver biopsy	HOMA-R values and insulin sensitivity indices did not correlate with steatohepatitis
El-Koofy *et al*[35]	18 patients with normal histology, 8 simple steatosis patients, and 7 NASH patients	2-15	Liver biopsy	HOMA-R values significantly differed between patients with normal histology and those with steatosis/NASH, and significantly correlated with grading based on US
Patton *et al*[36]	88 NAFLD patients	6-17	Liver biopsy	NASH *vs* not NASH: HOMA-R OR = 1.283 (*P*-value = 0.004) and QUICKI OR = 0.786 (*P*-value < 0.001)
Ko *et al*[37]	80 NAFLD patients (18 simple steatosis, 27 type 1 NASH, and 35 type 2 NASH)	10.4 ± 3.9, 12.6 ± 2.4, 12.3 ± 2.3, respectively	Liver biopsy	No differences in HOMA-R values between type 1 and type 2 NASH; HOMA-R values did not correlate with NAS
Manco *et al*[38]	82 NAFLD patients	3-18	Liver biopsy	HOMA-R and QUICKI values, and HOMA-beta secretion did not correlate with NAS
Nobili *et al*[39]	72 NAFLD patients	9-18	Liver biopsy	HOMA-R values did not correlate with NAS, steatosis, inflammation, ballooning, or fibrosis
Chan *et al*[40]	65 fatty liver patients	9.5-14	Liver biopsy and US	HOMA-R and QUICKI values correlated with severity of fatty liver evaluated by US. Higher insulin resistance significantly correlated with fatty liver severity only in male subjects with NASH

NAS: NAFLD activity score; US: Ultrasound; QUICKI: Quantitative insulin sensitivity check index; HOMA-R: Homeostasis model assessment as an index of insulin resistance; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; MRI: Magnetic resonance imaging.

been reported to decrease at a rate of 15% per year[29]. Significant correlations between insulin resistance and NAFLD activity scores (NAS), which were calculated by summing the scores for steatosis, lobular inflammation, and ballooning degeneration, were found in 177 children with NAFLD/NASH[30]. Adipose tissue insulin resistance is also present in the majority of adults with NAFLD, whether the patients are obese or not[31]. Reports in the literature on insulin resistance in pediatric NAFLD/NASH are summarized in Table 2[32-40]. These reports demonstrated that insulin resistance is associated with fatty changes using magnetic resonance imaging and ultrasound[32,40]. However, insulin resistance was not associated with fibrosis or NAS[32-40]. Therefore, these findings suggest that insulin resistance is important for the first hit in the two-hit model of NASH. In adults, insulin resistance did not correlate with NAS but correlated with fibrosis[41,42]. NASH in children is mainly characterized by fatty changes and fibrosis in the portal area (type 2 NASH), which is different to the characteristics of NASH in adults. Therefore, larger scale follow-up studies are required to understand the progression of NASH from children to adults.

CASES OF PEDIATRIC NAFLD/NASH ENCOUNTERED IN OUR DEPARTMENT

Table 3 summarizes the children with NAFLD/NASH that were treated in our department. The patients were 6-16 years old. Their ALT levels were generally high at 16-212 IU/L (normal range < 35 IU/L). Mean values of insulin and HOMA-R values were 23.5 (range: 11.7-272.2 μU/mL and 5.36 (range: 2.07-67.7), respectively. All cases were diagnosed by liver biopsy. All except 1 patient were compatible with type 4 NASH using Matteoni's criteria. The remaining case was type 3. The median NAS was 6 (range: 3-8). The median Brunt's inflammatory grade was 2 (range: 1-3). The median Brunt's fibrosis stage was 3 (range: 1-3). Five cases out of 12 were classified as grade 1, 2 cases were classified as grade 2, and 5 cases were classified as grade 3. The HOMA-R values did not correlate with NAS or Brunt grading.

GENETIC BASIS OF NAFLD/NASH

Familial clustering of NAFLD/NASH is obvious. Genetic predisposition as well as environmental factors including diet have been reported in NAFLD/NASH. Polymorphisms in the genes encoding *PNPLA3*, *UCP3*, *SLC2A1*, *Lipin1*, the *COX-2* promoter, and the *UCP1* (AG + GG) genotypes have been reported to be associated with the development of NAFLD. On the other hand, a genome-wide association study (GWAS) using liver mRNA from NAFLD patients showed that a combination of increased expression of lymphocyte cytosolic protein-1 (*LCP1*) and decreased expression of group-specific component (GC) is significantly associated with susceptibility to NAFLD/NASH. *GC* gene polymor-

Table 3 Pathology and homeostasis model assessment as an index of insulin resistance values of pediatric nonalcoholic steatohepatitis patients treated in our department

Patient number	Age (yr)	Matteoni's criteria	NAS	Brunt's grading	Brunt's staging	HOMA-R
1	6	4	7	3	2	40.6
2	9	4	4	2	2	2.72
3	11	4	6	2	3	4.60
4	11	4	6	2	3	5.83
5	12	4	7	3	3	3.65
6	13	4	5	2	3	58.5
7	14	4	5	2	2	20.0
8	14	4	7	2	2	3.36
9	14	4	8	2	3	3.95
10	14	4	3	1	3	67.7
11	15	4	6	2	2	4.89
12	15	4	7	2	3	17.3
13	16	3	7	2	1	19.4

NAS: Nonalcoholic fatty liver disease activity score; HOMA-R: Homeostasis model assessment as an index of insulin resistance.

Table 4 Efficacy of main drugs against nonalcoholic steatohepatitis/nonalcoholic fatty liver disease symptoms

	Drug	Efficacy
Insulin-sensitizing agent	[1]Metformin[47]	Controversial (effective but no more effective than improvement of lifestyle)
Antioxidants	[1]Vitamin E[47]	Significant improvements in NASH and NAFLD activity scores
	Vitamin C	No changes in ALT levels or liver inflammation; fibrosis was controlled intentionally
Liver-supporting drugs	Ursodeoxycholic acid	No improvements in serum transaminase and fat levels evaluated by US
	Phosphatidylcholine	No improvement in serum ALT level; improvements in liver echo intensity and insulin resistance
	[1]Taurine[48]	Decreased serum ALT levels and increased liver CT values in 7 children
Cholesterol-lowering agents	HMG-CoA reductase inhibitor (atorvastatin)	Decrease in serum ALT levels and improvement in liver pathology
	Probucol	Decrease in serum ALT levels

[1]Indicate drugs reported for children. US: Ultrasound; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; CT: Computed tomography; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; ALT: Alanine aminotransferase.

phisms and LCP1 levels are correlated with vitamin D levels and hyperlipidemia, respectively[43].

Genomic studies on patients with type 2 diabetes revealed some positive correlations of polymorphisms using GWAS. The correlation between gene single nucleotide polymorphisms (SNPs) in *PPAR-gamma*, *TCF7L2*, *G6PC2*, *MTNR1B*, *etc*., have been reported in adolescents as well as in adults[44,45]. In particular, gene SNPs in *TCF7L2*, *IGF2BP2*, *CDKAL1*, *HHEX*, and *HNF1A* might be associated with a higher risk of type 2 diabetes in obese children and adolescents[46]. These genes are involved in the release of insulin granules from beta cells.

MANAGEMENT OF PEDIATRIC NASH AND NAFLD

NAFLD is often associated with obesity, diabetes, hyperlipidemia, and hypertension, and is considered to be a type of metabolic syndrome.

Because NASH is considered to progress from fatty liver, the management of fatty liver is important. Progressive increases in intrahepatic TG levels are associated with progressive impairment of insulin action in skeletal muscle and adipose tissue, in addition to the liver[30]. The principles of treatment are to make improvements in lifestyle, such as diet and exercise. In adults, treatments to improve insulin resistance and oxidative stress have been attempted. The efficacy of insulin sensitizers and antioxidants has also been reported, but there are no established treatments to date.

Quick weight loss can also worsen liver fibrosis. Children with NAFLD often become treatment dropouts, and a relapse is observed in more than 90% of these children. The efficacy of drugs from reports in the literature is shown in Table 4. However, these reports are limited to children[47,48]. In many cases, transaminase levels can be normalized by weight loss of approximately 5%.

The prognosis of NASH in adults is still obscure. Previous studies reported that 5%-20% of patients develop liver cirrhosis within 5-10 follow-up years. Liver re-biopsy within 3-6 years revealed that 40%-50% of patients showed no change, 30%-50% worsened, and 20%-30% improved[49]. AST and ALT levels and disease progression sometimes do not correlate, particularly if there are no subjective symptoms. 10%-20% of the patients showed liver cirrhosis.

A long history of lifestyle-related diseases, severe obesity, type 2 diabetes, low platelet count, rise in fibrosis

markers (hyaluronic acid and type Ⅳ collagen 7S), and liver dysfunction are assumed to affect NASH-associated liver cirrhosis. There are no large-scale studies on childhood NASH, and the prognosis is unknown. Therefore, careful evaluation of fibrosis should be performed during their follow-up.

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MINIREVIEWS

Effect of periodontal treatment on adipokines in type 2 diabetes

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Abstract

The association between adipokines and inflammatory periodontal diseases has been studied over the last two decades. This review was intended to explore the observation that periodontal therapy may lead to an improvement of adipokines in diabetic patients. In summary, substantial evidence suggests that diabetes is associated with increased prevalence, extent and severity of periodontitis. Numerous mechanisms have been elucidated to explain the impact of diabetes on the periodontium. However, current knowledge concerning the role of major adipokines indicates only some of their associations with the pathogenesis of periodontitis in type 2 diabetes. Conversely, treatment of periodontal disease and reduction of oral inflammation may have positive effects on the diabetic condition, although evidence for this remains somewhat equivocal.

Key words: Adipokines; Diabetes; Periodontal disease; Periodontal therapy

Core tip: Several adipokines could serves as the monitoring molecules that reflect overall and oral disease conditions include periodontitis. Because they are rapidly change upon the change in body and oral conditions. The treatment response and disease activity progression may also predicted using these kinds of molecules. Moreover, the method to collect and analyse adipokines is relatively simple because they can be detected in gingival crevicular fluid and analysed using general enzyme-linked immunosorbent assay technology. Collectively, clinicians include medical doctors and periodontists should take the concern regarding adipokines into their routine periodontal treatment plan and management.

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OVERVIEW OF PERIODONTITIS AND INFLAMMATION IN TYPE 2 DIABETES

Periodontal disease refers to the processes of destruction of the peri-tooth structures that support the teeth. These comprise the gingiva, the periodontal ligament, the cementum and the alveolar bone. The chronic destruction of these supporting tissues leads to the eventual loss of teeth. Epidemiological studies have revealed that more than two-thirds of the world's population suffers from

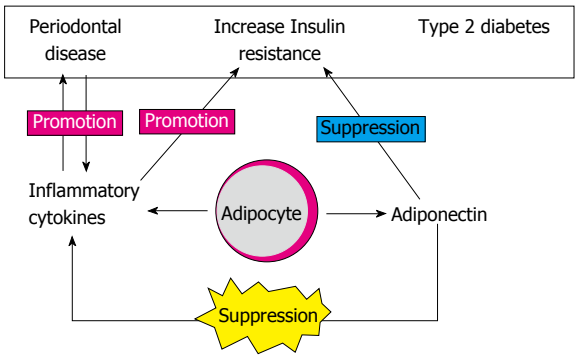

Figure 1 Relationship between type 2 diabetes and periodontal disease (hypothesis).

one of the chronic forms of periodontal disease[1].

Periodontal destruction is host-mediated by locally produced pro-inflammatory cytokines in response to the bacterial flora and its products[2]. It is possible that the production of local cytokines[3] and or low-level asymptomatic bacteremia or endotoxemia[4] affects the plasma concentration of pro-inflammatory biomarkers.

Significant differences in the plasma concentrations of such biomarkers have been described[5-8]. Periodontitis may have an even greater influence on the systemic inflammatory condition in individuals with diabetes. Elevated circulating levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and high-sensitivity C-reactive protein, which can worsen insulin resistance and thereby impair glycemic control, have been shown in several studies[9,10]. Thus, periodontal disease may have a significant impact on the metabolic state in diabetes[11]. TNF-α has been reported to play a key role in the pathogenesis of type 2 diabetes, and the correlation of this cytokine with insulin resistance has also been shown in metabolic syndrome[12].

Several studies have reported the effects of periodontal treatment on glycemic control as well as systemic inflammatory mediator levels in patients with type 2 diabetes. In some cases, positive effects such as improving HbA1c or serum level of adiponectin have been indicated[13,14]; however, such phenomena regarding adipokines are still unclear due to several confounding factors. Adipokines are molecules mainly produced and exocytosed from adipocytes. These molecules are a large family composed of members such as leptin, adiponectin, resistin, visfatin, adipsin, interleukin, monocyte chemotactic protein-I and retinol-binding protein.

Accordingly, this review focuses on providing a concise summary and dealing with recent advances regarding the potential of selected adipokines as therapeutic tools or targets of periodontal treatment (Figure 1).

ADIPOKINE MOLECULES AND PERIODONTAL TREATMENT

Leptin

Leptin, a molecule that acts as an obesity-regulatory hormone, has the cytogenetic location of 7q32.1[15]. The gene encoding leptin is named the *LEP* gene or the obese gene, which produces a 16-kDa protein secreted by white adipose tissue. By interaction with leptin receptor[16], it leads to appetite regulation, control of body energy expenditure and maintenance of bone mass. The actions of leptin mainly occur in the hypothalamus[17]; however, the production of leptin has also been found in bone marrow, placenta, skeletal muscle and stomach[17-20]. Recently, it has been found that leptin could reduce adipose tissue inflammation *via* activation of the macrophage histone deacetylase HDAC4[21]. In an animal model, namely, mice without the *LEP* gene, which are dramatically obese, leptin injection led to weight loss due to food intake reduction and increased energy expenditure[16,22].

The relationship between leptin and insulin is still not well established. At present, it has been demonstrated that leptin suppresses insulin production *via* a negative feedback loop, but insulin stimulates the production of leptin[23,24]. These interplays occur in an axis named the adipo-insular axis, and progression of insulin resistance was shown to be correlated with dysregulation of this axis[25]. Recent evidence in an *in vitro* model has demonstrated that leptin influenced insulin by regulation of insulin-like growth factor-binding protein 2[26], and this regulation occurred through signal transducers and activators of transcription (STATs), especially STAT-3, as well as phosphatidylinositol-3-kinase and the Akt signaling pathway[26,27].

Leptin and periodontal treatment

Inflammation of periodontal tissue results in an increased serum leptin level, but leptin significantly decreased ($P < 0.05$) during a 3-mo follow-up period in type 2 diabetic patients who received non-surgical periodontal treatment[28]. Even though this study and a study by Teres *et al*[29] found that leptin correlates with inflammatory condition because they found a positive relationship between IL-6 and leptin but a negative relationship between vitamin D and IL-6, the latter study failed to show that periodontal therapy could change the level of leptin as well as those of other adipokines in serum. Recent evidence has also suggested that the combination of periodontal treatment with periodontal antibiotic treatment could improve the periodontal status of Japanese type 2 diabetic patients without dramatically affecting the serum leptin level[30]. From all of the above studies, it seems that leptin is not a sensitive marker for periodontal tissue change or improvement. This molecule may reflect the systemic inflammatory conditions rather than local ones.

Adiponectin

Adiponectin (also known as Acrp30, apM1 or GBP28) is a 3-kDa adipokine secreted mainly by adipocytes, which plays important roles in the homeostasis control of glucose, energy and lipid metabolism. The adiponectin gene (*Adipoq*) is located on chromosome 3 at 3q27[31]. Although this protein is secreted mainly by adipocytes, it is also

secreted by other cell types include cardiomyocytes[32,33]. Unlike other adipokines, adiponectin exerts anti-inflammatory, anti-diabetic as well as anti-arthrogenic activities[34-36]. Attempts have been made to utilize this molecule as a therapeutic agent or for obese patients. Adiponectin exerts its activity *via* two types of receptor, namely, adiponectin receptor 1 (ADIPOR1) and ADIPOR2[37]. Both of these are widely expressed in diverse cell types, include cardiovascular and immune cells. ADIPOR1 is expressed markedly in skeletal muscle cells, whereas ADIPOR2 is expressed mainly in liver cells[37,38]. When adiponectin binds to its receptor, the signaling pathway *via* activation of peroxisome-proliferator-activated receptor-γ, AMP-activated protein kinase (AMPK) or p38 mitogen-activated protein kinase (MAPK) has been shown to be active[27]. Among these, AMPK acts as a major downstream molecule of the adiponectin signaling pathway[39].

Chronic low-grade inflammation and oxidative stress in obesity have been shown to downregulate *Adipoq* gene and protein expression[40]. TNF-α and IL-6, two main inflammatory molecules, are capable of downregulation of adiponectin *via* protein kinase C[41] and MAPK signaling[42], respectively. Moreover, adiponectin inhibits monocyte adhesion to endothelial cells as well as inhibiting macrophage function, collectively contributing to inflammatory cascade regulation[43]. In addition, adiponectin was shown to significantly induce anti-inflammatory cytokines ($P < 0.05$), for instance, IL-10 and IL-1 receptor antagonist, in human monocytes and macrophages[44]. Recently, it was also found that adiponectin could induce the pro-inflammatory function of isolated CD4+ T cells and macrophages by enhancing T-cell differentiation and the induction of interferon gamma production[45]. This suggests a new role of adiponectin in the induction of selected inflammatory stimulation for desensitizing these cells to further stimuli.

In liver, adiponectin reduces gluconeogenesis in concert with insulin and improves insulin sensitivity[46,47]. The plasma level of adiponectin in isolated human subjects is also inversely related to fasting insulin level ($r = -0.63$) and insulin resistance ($r = -0.38$)[48]. From these lines of evidence, adiponectin has been studied for the possibility of using it as a target for diabetic drugs, especially in type 2 diabetes, and also in cardiovascular diseases.

Adiponectin and periodontal treatment

In elderly patients with chronic periodontitis, serum adiponectin level is similar to that in periodontally healthy subjects, but females have a higher serum adiponectin level than males[49]. In addition, non-surgical periodontal treatment given to adult patients with mild to moderate periodontitis did not affect the serum adiponectin level[29]. This may be explained by the fact that adiponectin has different isoforms (low, middle and high molecular weight)[50] with different functions. In addition, it was suggested that only the ratio of high-molecular-weight adiponectin to total adiponectin was significantly lower in subjects with periodontitis[51]. Furthermore, diabetic patients with periodontitis who received periodontal treatment without or with topical antibiotics showed significant elevation of serum adiponectin compared with an untreated group ($P < 0.05$)[28,30]. Effective control of inflammation by periodontal treatment with local antibiotics may contribute to increase systemic anti-inflammatory markers such as adiponectin and hence improve overall health status[14].

Resistin

Resistin [also known as adipocyte-specific secretory factor and found in inflammatory zone (FIZZ)] is a 12.5-kDa protein said to play a role as a mediator of insulin resistance[52]. The name resistin comes from the finding that this molecule provides resistance to insulin. The gene that encodes this molecule, named *Retn*, is located on chromosome 19 at p13.3[53]. Interestingly, in humans, resistin is predominantly secreted by macrophages, rather than adipocytes[54]. Bone marrow, peripheral mononuclear cells, lung[55], placenta tissue[56] and pancreatic β-cells[57] can also express this molecule. Murine adipocytes, when cultured in the presence of insulin-sensitizing drugs, for example, thiazolidinediones, appeared to exhibit suppressed resistin secretion[53]. Circulating resistin was shown to decrease upon the administration of anti-diabetic drugs such as rosiglitazone, and to be increased in diet-induced and genetic forms of obesity. From these lines of evidence, it has been postulated that resistin may function as a link between obesity and diabetes, especially type 2 diabetes. However, one study did not find any relationship between resistin and obesity or insulin resistance[54]. This controversial finding may be explained in part by the fact that resistin has at least 2 isoforms: a high-molecular-weight hexamer form and a more bioactive but less prevalent low-molecular-weight trimer form, which exerts a different biological function[27,58]. Numerous clinical studies have demonstrated a possible relationship of resistin and insulin resistance in obese people with or without diabetes. The possible contributing factor that links resistin to insulin resistance may be hyperresistinemia. In addition, recent clinical studies have shown that individuals with a high serum resistin level have a significantly increased risk of developing type 2 diabetes[59,60].

Resistin may play a pivotal role in monocyte-macrophage function and inflammation due to the finding that the expression of resistin was increased in concert with the maturation of monocytes into macrophages[55]. At present, the concrete mechanism of resistin-mediated inflammation has not yet been established due to the resistin receptor not being identified yet, but an isoform of decorin and tyrosine kinase-like orphan receptor 1 were proposed as functional resistin receptors that may modulate glucose homeostasis or regulate enlargement of white adipose tissue in rodents[61,62]. Many pro-inflammatory stimuli and cytokines including lipopolysaccharide, TNF-α, IL-6 and IL-1β are capable of inducing resistin expression and function[63-65]. One line of evidence suggested that resistin could also induce the secretion of pro-inflammatory cyto-

kines, for instance, TNF-α, IL-6, IL-12 or monocyte chemoattractant protein-1 in peripheral blood mononuclear cells and macrophages[65,66]. Collectively, these findings show that resistin is a molecule that is closely related to systemic inflammation.

Resistin and periodontal treatment

The relationship between serum resistin and periodontal condition was investigated by Furugen *et al*[49], who found that serum resistin and total leukocyte count in subjects with periodontitis were higher than those in subjects without 6-mm pocket depth or without bleeding on probing, with an odds ratio of 2.0 or more. Saito *et al*[67] also found an association between increased severity of periodontitis and increased serum resistin level both in bivariate (OR = 3.0; 95%CI: 1.2-7.6) and multivariate analyses (adjusted OR = 3.1; 95%CI: 1.1-8.6) analyses, and concluded that the increased levels of serum resistin in middle-aged women might affect their systemic health. After non-surgical periodontal treatment, the serum resistin level in periodontitis patients who have no underlying disease decreased to some extent[68]. Recently, periodontal treatment with antibiotics in type 2 diabetic patients was shown to result in no difference of serum resistin level compared to that of healthy counterparts[30]. However, this study was performed in only a small number of subjects (21 subjects) and all subjects were categorized into mild periodontitis. The effect of periodontal treatment on serum resistin needs to be more clearly elucidated in a larger sample.

Visfatin

Visfatin, a 52-kDa protein, is another adipokine secreted by adipocytes and mimics the effect of insulin[69]. This molecule was found to be enriched in visceral adipose tissue, which is the reason for its name. It was also known as pre-B-cell colony-enhancing factor (PBEF)[27] or nicotinamide phosphoribosyltransferase (Nampt)[70] PBEF or Nampt, with the gene located on chromosome 7 at q22.3[71]. Visfatin is essential for nicotinamide adenine dinucleotide biosynthesis and hence is related to cell metabolism. In humans, visfatin is mainly expressed in bone marrow (highest expression in leukocytes), liver and muscle cells. It is also expressed in various tissues, including heart, lung, kidney and placenta. Visfatin has 2 isoforms: intracellular and extracellular ones. The intracellular isoform mainly functions in energy production in cells, while the extracellular isoform is related to increased inflammatory cytokines, such as TNF-α, IL-1β, IL-16 and transforming growth factor-β1, and the chemokine receptor C-C chemokine receptor type 3[72].

Visfatin has insulin-mimicking effects, for example, increasing glucose uptake and enhancing triglyceride biosynthesis, because it binds to the insulin receptor, although at a different site from insulin[69]. In type 2 diabetic individuals, it was demonstrated that visfatin impaired vascular endothelial function as well as creatinine clearance[73], which probably leads to atherosclerosis and chronic kidney disease. Additionally, the visfatin level in this type of patient was found to be enhanced, which positively correlated with increased homocysteine, an endothelial dysfunction marker[74]. It seems that visfatin levels are positively associated with a series of inflammatory conditions, independently of other potential metabolic implications[75].

Research has mainly focused on the role of visfatin in cardiovascular diseases. As mentioned earlier, it was shown to induce inflammation of endothelial cells and vascular smooth muscle cells. It also induced TNF-α and IL-8 production from peripheral mononuclear cells[76]. Additionally, macrophage survival was promoted by visfatin[77]. Exogenous visfatin could stimulate inducible nitric oxide synthase, which is a pro-inflammatory cytokine that contributes to endothelial dysfunction and vascular injury in diabetes-related vascular complications[78,79].

Visfatin and periodontal treatment

Because visfatin exerts pro-inflammatory functions in several organs, this molecule also correlates with chronic inflammation of periodontal tissue. In periodontitis, it was reported that visfatin concentration was increased in such patients and the more severe the periodontitis, the higher the level of visfatin observed in serum and gingival crevicular fluid (GCF)[80]. Another study was performed on an observational basis in healthy subjects, those with periodontitis without diabetes and those with periodontitis with diabetes; it was found that the mean visfatin in both serum and GCF was markedly increased in diabetic patients concurrently burdened by periodontitis[81]. The periodontal ligament cells could produce visfatin and *Fusobacterium nucleatum*, one of the periodontopathic bacteria, enhanced the level of visfatin, which supports the assertion that bacteria exert an inflammatory bioburden on periodontal tissue. This effect could be reversed by biomechanical loading[82]. The effect of non-surgical periodontal treatment on serum and GCF visfatin level in periodontitis patients was reported by Raghavendra *et al*[83], who found that periodontal treatment given to periodontitis patients could decrease a high visfatin level in the active disease stage to a nearly normal level, as in periodontally healthy individuals both GCF ($P < 0.001$) and serum ($P = 0.008$). Although no study has yet been conducted on the effect of non-surgical periodontal treatment on the level of visfatin in periodontitis patient with diabetes, it seems that this molecule is associated with inflammatory conditions and can be used as an inflammatory marker or periodontal disease activity marker at both local and systemic levels.

Adipsin

Adipsin, also known as complement factor D, factor D and adipocyte trypsin, is one of the adipokines secreted by adipocytes into the bloodstream. The adipsin gene in humans is located at p13.3 on chromosome 19[84]. Adipsin belongs to the serine protease family and functions in cleavage of the bond between complement factor 3

and factor B[85]. Human adipsin is a 24-kDa molecule that stimulates acylation-stimulating protein and is then involved in the stimulation of glucose transport, enhancement of fatty acid re-esterification and facilitation of lipid lipolysis[86]. In humans, plasma levels of adipsin are not different or slightly increased in the obese population compared with the non-obese one[87,88], but this remains controversial. Recently, it has been demonstrated *in vitro* that high glucose promoted adipocyte-derived molecules including adipsin and resistin, but inhibited osteogenic differentiation in osteosarcoma (MG-63) cells[89]. Recently, adipsin level was increased and positively correlated with lung fibrosis ($r = 0.412$, $P < 0.001$) and pleural plaque ($r = 0.245$, $P = 0.043$), in asbestos-exposed workers[90]. This suggested the role of adipsin in inflammation enhancement.

Adipsin and periodontal treatment

Concerning the role of adipsin in periodontitis, it was suggested that it exerted the same activity as *P. gingivalis*, resulting in the breakdown of periodontium[91]. The effect of periodontal treatment on the change of adipsin in human subjects has not been reported yet, but we hypothesize that this molecule might be decreased as a result of inflammatory reduction after periodontal therapy.

PERSPECTIVES

Adipokines are much more complex and involved in many systems, include immune and endocrine systems, and these molecules influence the pathogenesis of obesity-related diseases, particularly type 2 diabetes and cardiovascular diseases, as well as inflammatory diseases, especially periodontitis. A growing number of molecules have been identified to be secreted from adipocytes and more are yet to be discovered. Unravelling their orchestrated roles in controlling obesity, inflammation and periodontal health may lead to successful management of pathological conditions. Some markers, especially visfatin, are molecules that are closely related to inflammation, diabetic condition and periodontitis. With the recent development of sophisticated means to study molecules, we now aim to detect, analyze and make use of a number of molecules simultaneously to screen, explain and monitor the therapeutic outcome of disease conditions. This is due to no single molecule being able to reflect the nature of complex multifactorial diseases such as periodontitis and diabetes. Thus, the disease profile should be set as a template from several integrated adipokines, not only quantitatively for each molecule but also qualitatively. Here, single-nucleotide polymorphisms of each gene controlling these adipokines should be taken into account for periodontitis staging in diabetic patients and evaluating the disease response.

Not only data from serum but also data from non-invasive methods, for instance, analyses of gingival crevicular fluid and saliva, should be utilized as robust confirmation of local periodontal health. An ideal marker for periodontitis will not only demonstrate a clear relationship with periodontitis, but also be linked to systemic conditions that are influenced by periodontitis. To develop an adipokine candidate to use as a periodontal disease-specific biomarker or therapeutic compound, we also need to perform experiments mainly in human subjects to complete our understanding of the mechanism of such substances.

Robotic science has emerged as an important field in medicine. In the next century, *in vitro* robot-assisted synthesis of therapeutic molecules that combines the advantages of each adipokine will probably be launched on the market and make a major contribution to the treatment of severe periodontal breakdown, more effectively than contemporary therapeutic modalities. At that time, periodontitis in diabetic patients may no longer be a major oral health problem.

CONCLUSION

Current knowledge concerning the roles of major adipokines provides only a partial understanding of their associations with the pathogenesis of periodontitis in type 2 diabetes. This is probably due in part to the limited number of studies conducted on an acceptable number of human subjects. More studies regarding the effect of periodontal therapy on several adipokines should be performed. Nevertheless, we saw potential to develop visfatin as a tool for drug discovery and to generate more specific therapeutic targets. A novel cocktail of adipokine-related therapeutic strategies may offer opportunities for the successful management of periodontitis concomitant with diabetes.

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MINIREVIEWS

Risk factors for mortality in children with diabetic keto acidosis from developing countries

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Abstract

Diabetic keto acidosis (DKA) is the major cause for mortality in children with Diabetes mellitus (DM). With increasing incidence of type 1 DM worldwide, there is an absolute increase of DM among children between 0-14 year age group and overall incidence among less than 30 years remain the same. This shift towards younger age group is more of concern especially in developing countries where mortality in DKA is alarmingly high. Prior to the era of insulin, DKA was associated with 100% mortality and subsequently mortality rates have come down and is now, 0.15%-0.31% in developed countries. However the scenario in developing countries like India, Pakistan, and Bangladesh are very different and mortality is still high in children with DKA. Prospective studies on DKA in children are lacking in developing countries. Literature on DKA related mortality are based on retrospective studies and are very recent from countries like India, Pakistan and Bangladesh. There exists an urgent need to understand the differences between developed and developing countries with respect to mortality rates and factors associated with increased mortality in children with DKA. Higher mortality rates, increased incidence of cerebral edema, sepsis, shock and renal failure have been identified among DKA in children from developing countries. Root cause for all these complications and increased mortality in DKA could be delayed diagnosis in children from developing countries. This necessitates creating awareness among parents, public and physicians by health education to identify symptoms of DM/DKA in children, in order to decrease mortality in DKA. Based on past experience in Parma, Italy it is possible to prevent occurrence of DKA both in new onset DM and in children with established DM, by simple interventions to increase awareness among public and physicians.

Key words: Diabetic keto acidosis; Mortality; Cerebral edema; Sepsis; Shock; Delayed diagnosis

Core tip: Mortality in Diabetic keto acidosis (DKA) among children from developed countries is due to cerebral edema and is very low. The mortality in DKA among children from developing countries is due to higher incidence of cerebral edema, sepsis, shock and renal failure. Delayed diagnosis is the root cause for high mortality in children with DKA from developed countries. There is an urgent need to increase the awareness about diabetes among the public and physicians.

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INTRODUCTION

Diabetes mellitus in children is on the rise for past few decades. On an average 78000 children are diagnosed with diabetes every year[1]. One among every five children with newly diagnosed type 1 diabetes mellitus (DM) is

found to be an Indian[1]. In this world pandemic of diabetes with efforts to control type 2 DM it is easy that the needs of type 1 DM who are only 10% of people with diabetes is forgotten. Occurrence of type 1 DM is on the rise among children between 0 and 14 years of age. Majority of children present with diabetic keto acidosis (DKA) at onset and this rate is inversely proportional to prevalence of DM in the population[2]. Death in DM is predominantly due to DKA. Mortality rates in developed countries and developing countries show much variation. Similarly the cause for mortality in DKA varies between developed countries and developing countries. Cerebral edema is the predominant cause for mortality in children with DKA from developed countries, while recent data from developing countries has shown higher incidence of cerebral edema, sepsis, shock and renal failure as the cause for death in DKA[3]. Delayed diagnosis has been identified as a major risk factor associated with mortality in children from Chennai-India[3].

Overall mortality in children with DKA varies from 0.15% to 0.35% in developed countries like Canada, United States and United Kingdom[4-7] and from 3.4% to 13.4% in developing countries like India, Pakistan and Bangladesh[8-14]. Cerebral edema is the major cause for mortality in DKA[15,16]. Occurrence of cerebral edema varies from 0% to 5.5% in developed countries[17-19] and is reported to vary from 24%-26% in developing countries[10]. Literature on reasons for such high mortality and associated factors for death in children with DKA in developing countries are very recent and majority of these are based on retrospective studies. Whether factors associated with mortality are pre hospital in nature or treatment related needs to be understood. In the editorial published in Indian Pediatrics during the year 2004, titled "What determines the outcome of DKA in children from a developing country?" author has raised issues regarding fluid therapy in DKA[20]. The role of amount and rate of fluid administration in the management of DKA associated cerebral edema is still controversial. Traditionally cerebral edema has been linked to fluid therapy in DKA. A recent article titled 'Warning from India' has addressed the issue of high mortality and high incidence of sepsis and cerebral edema in children with DKA from a developing country[21]. Association of sepsis may have a great impact on fluid therapy in DKA.

DKA RELATED CEREBRAL EDEMA

Cerebral edema has been the major risk factor for mortality in children with DKA world over. Despite decades of management of DKA the exact cause for cerebral edema in DKA is yet to be understood. Whether hypo perfusion related ischemia leading to cytotoxic edema or reperfusion induced vasogenic edema, is the cause for cerebral edema is controversial. However initial cytotoxic edema followed by subsequent vasogenic edema can very well contribute to development of cerebral edema in DKA. Also the role of inflammatory mediator release, glucotoxicity, uremia or acidosis in causing cerebral edema, is not clearly understood. Occurrence of cerebral edema can be at the time of presentation or during therapy up to initial 24 h. Predisposing factors for cerebral edema in children with DKA have been identified in various studies in developed countries. Identified factors are disease related or treatment related or both. Identified factors vary from young age at presentation, new onset disease, rate and amount of fluids used for resuscitation, blood urea nitrogen, body mass index, initial osmolality, rapid fall in osmolality, failure of sodium to rise with treatment, use of bicarbonate for correction of acidosis, insulin infusion in the first hour of therapy of DKA or bolus insulin therapy in DKA[22-30]. There has been no consistency among the factors identified for occurrence of cerebral edema in various studies published till date.

Occurrence of cerebral edema from developing countries has been found to be as high as 26% among a cohort of children admitted at a pediatric intensive care unit in north India[11]. Literature on reasons for such high incidence of cerebral edema from developing countries is very scarce. Studies by Tiwari *et al*[11] from Chandigarh-India have identified fluid refractory shock, higher volume of fluids at admission and respiratory failure requiring ventilation to be significant risk factors for cerebral edema in DKA. However only fluid refractory shock, azotemia and younger age were identified to be significant risk factors for cerebral edema in multivariate analysis[11]. Literature from Chennai-India has revealed cerebral edema in 24% of study group[3]. In this prospective study of 118 children with DKA, specific risk factor related mortality for cerebral edema was 43%. A higher fluid bolus at the emergency room for resuscitation was a significant therapy related factor for cerebral edema by univariate analysis. Cerebral edema was significantly associated with altered sensorium, lower $PaCO_2$ at admission, delayed diagnosis and failure of sodium to rise with therapy by multivariate analysis. Both the studies from India have identified higher fluids as risk factors for cerebral edema in univariate analysis but were not significant in multivariate analysis[3,11]. This may be an important observation in developing countries where sepsis has been an important factor associated with increased mortality in children with DKA. Too much of fluid for resuscitation resulting in cerebral edema is still controversial in DKA. Similarly less fluid in a child with DKA and shock may also worsen risk of cerebral edema and renal failure. Sepsis by itself will demand large volumes of fluid boluses in a child. Hence recommendations regarding fluid therapy based on guidelines from developed countries where sepsis and shock are not major factors in children with DKA needs to be addressed for future guidelines when applied to developing countries. Whether there is a need for more liberal fluid therapy in DKA in developing countries where sepsis, shock and renal failure have been identified to be risk factors for mortality needs to be addressed by multicentric trials.

SEPSIS IN DKA

Sepsis in DKA as a risk factor for increased mortality has been identified in studies form developing countries like India, Pakistan and Bangladesh[8-12,14]. Majority of these studies were based on retrospective data. Still they have identified sepsis as a definite risk factor for mortality in children with DKA. Though infections have not been identified to be a major comorbid state in children with type 1 diabetes from developed countries, studies from Chennai-India has shown that infections are much more common in children with diabetes in comparison to children without diabetes[31]. In this context infections do play a major role in children with DKA. Sepsis not only precipitates DKA, also complicates fluid therapy, predisposes to renal failure and is associated with increased mortality in DKA based on data from developing countries. Jayashree *et al*[10] in 2004 from India published their retrospective study in DKA. They reported that among 64 children with DKA 30 children had foci of infection. Respiratory infection in 10, soft tissue infection in 10, meningitis in 3, hepatitis in 2, peritonitis, chronic suppurative otitis media, tonsillitis, ethmoiditis and oral and vulval candidiasis in one each. Cerebral edema and complicating sepsis were reported to result in poor outcome in children with DKA. In their series, sepsis was the triggering factor in one third of cases. In study from Chennai-India, infections were encountered in 61 children among the study group of 118 children[3]. Of these 49 had identified focus of infection (41.5%). Culture positive sepsis was seen in 12% of children with DKA and is associated with specific risk related mortality of 57%. Other infections encountered were pneumonia, urinary tract infections, skin and soft tissue infections, mucormycosis, acute suppurative otitis media, enteric fever and peritonitis. Kanwal *et al*[12] from Delhi India has identified 32.7% of study group (18 of the 55 children) to have sepsis. Documented infection were reported to be 16.3%. Urinary tract infection, pneumonia, diaorrhea and culture positive sepsis were the identified infections. Study by Tiwari *et al*[11] published in 2012 had revealed 58% of study population as sepsis as per standard definition. However only 1/5th of this group had a focus of infection identified. Respiratory tract was the focus in 6, gastrointestinal in 4, sinonasal mucormycosis, urinary tract infection (UTI), acute otitis media, peritonitis, tonsillitis and cellulitis one each. Infections have been reported in 48% of children with DKA by Zabeen *et al*[14] from Bangladesh. Mortality in their study group were attributed to cerebral edema and sepsis. Respiratory infections were commonest followed by urinary tract infections, sepsis and pneumonia.

Studies from Iran by Asl *et al*[32] reported that among 63 children with DKA 13 of them had infections. This was inclusive of pneumonia, tuberculosis, diarrhea and upper respiratory infections. The study documented acute renal failure in 4.7%. Clinical diagnosis of sepsis as well as shock may be over diagnosed in children with DKA. Presence of fever in DKA signifies infection and the focus need to be identified. The criteria for systemic inflammatory response (SIRS), when applied to children with DKA may lead to over diagnosis of sepsis. Tachycardia and tachypnea as criteria can be explained by dehydration and keto acidosis rather than sepsis and lactic acidosis. Lactic acidosis in DKA could be due to sepsis, hypovolemia or due to disturbed carbohydrate metabolism perse. Similarly DKA is known to be associated with leucocytosis and this is not specific for sepsis in DKA[33]. Leukocytosis is a part of stress response in DKA and may be seen in up to 50%-60% of children with DKA[34]. One needs to be very cautious about diagnosing sepsis based on the criteria for SIRS in DKA. Any child with fever or a focus of infection along with any of the above criteria can be taken as sepsis complicating DKA. Current guidelines from developed countries where sepsis is not a major factor, do not recommend antibiotics in DKA. Based on literature evidence from developing countries, sepsis is more common and sepsis complicating DKA has increased mortality. Hence antibiotics may be empirically considered in children with fever or refractory shock despite the absence of obvious focus of sepsis, until infections have been ruled out in DKA among children from developing countries.

SHOCK IN DKA

Shock as a presentation in DKA is rare in literature from developing countries[35]. International Society for Pediatric and Adolescent Diabetes clinical practice consensus guidelines 2009 compendium states the following "Despite of their dehydration, patients continue to maintain normal blood pressure and have considerable urine output until extreme volume depletion and shock occurs, leading to a critical decrease in renal blood flow and glomerular filtration"[36]. However it is uniformly reported in literature from developing countries that shock at presentation in children with DKA is fairly common. Studies from Pakistan[8] have revealed incidence of shock to be 19.3% in their study and overall mortality was 3.4%. Tiwari *et al*[11] from Chandigarh, India documented in their study that 48% of study population with DKA at the pediatric intensive care unit had hypotensive shock at presentation and of them 30% needed inotropes. Kanwal *et al*[12] from India have documented in their study on 55 DKA children, incidence of shock to be 18.1%, 10.9% were due to hypovolemia and 7.25% were due to septic shock. Study from Chennai[3] has shown occurrence of shock at presentation in DKA to be 12% and specific risk factor related mortality in DKA to be 53%. According to another study from Chennai, India among the 23 children with DKA 10 presented with shock[37]. However criteria used to assess shock in those children and severity of shock had not been discussed. Shock in DKA is a combination of hypovolemia and sepsis. To differentiate between the two is difficult and most of the time it may be a combination of hypovolemia and sepsis. The clinical evidence for hypovolemia in DKA is not reliable

as published in literature. Intra cellular dehydration in DKA may not be clinically evident and hence degree of dehydration may be under diagnosed. Capillary refill time in DKA cannot be relied as a sign of shock in DKA[38]. Tachycardia could be a physiological response to dehydration in DKA and this needs caution while interpreting it as a sign of shock. Tachypnea for similar reasons is due to acidosis which is predominantly keto acids and cannot be interpreted as a sole evidence of hypo perfusion and lactic acidosis. Altered sensorium in DKA can be explained by cerebral edema, severe acidosis or shock in DKA. This feature cannot be relied as a sign of poor end organ perfusion of shock. In developing countries where cerebral edema, shock, sepsis and renal failure are reported to be common in DKA, diagnosis based on clinical features alone may be challenging for the pediatrician at the emergency department. Hence the criteria for septic shock or hypovolemic shock may need to be applied with clinical judgment in children with DKA. Presence of fever, hypotension, wide pulse pressure in septic shock, clinical evidence of dehydration in hypovolemia may be better indicators of type of shock in DKA. Similarly is the assessment of dehydration in shock. Clinical signs of dehydration may not be evident in DKA. Since initial dehydration is predominantly intra cellular there may not be obvious clinical evidence of dehydration at presentation in a child with DKA. This might lead to underestimation of degree of dehydration in DKA. With recent literature from developing countries regarding shock and sepsis in DKA, we need to reappraise the existing guidelines from developed countries for fluid therapy in children with DKA. Whether less fluid is harmful or more fluid is harmful needs to be answered by well planned fluid trials for children with DKA from developing countries.

RENAL FAILURE IN DKA

Renal failure in children with DKA is a complication unheard of in literature from developed countries[39]. Children from developing countries presenting with renal failure in DKA is not uncommon. Studies from Iran by Asl *et al*[32] reports that 4.7% of children with DKA had acute renal failure. Studies from Bangladesh by Zabeen *et al*[14] have shown the incidence of renal failure to be 3.7% in DKA. Published literature from Chennai, India[40] revealed acute renal failure in DKA to be 11.5%. Mortality among children with DKA and acute renal failure was documented to be 40%-72%. Sepsis, shock and rhabdomyolysis causing acute renal failure have been reported in the series. Renal failure leads to difficulty in diagnosis as well as management of DKA. Oliguria and anuria as criteria for renal failure is not reliable in DKA due to osmotic diuresis of hyperglycemia. Similarly, urea and creatinine values may be elevated in DKA due to prerenal causes like dehydration which declines with adequate fluids. Subsequent elevation in creatinine cannot be taken as a definite criterion for renal failure as the commonly used calorimetric method of creatinine estimation is likely to be associated with spurious elevation due to interference by ketones. Fluid restriction in a child with sepsis and shock (hypovolemic or septic) for fear of cerebral edema during management of DKA may predispose to renal failure. Child with severe dehydration with delay in diagnosis may present with acute tubular necrosis leading to renal failure in DKA. Management of renal failure in DKA poses great difficulty for the treating physicians. Following needs to be considered in renal failure in DKA-modification of amount and type of fluids for therapy, consideration of using bicarbonate, varied metabolism and sensitivity of insulin. Peritoneal dialysis in such children also leads to severe fluctuations of blood glucose levels. Presently there are no standard guidelines for management of renal failure in DKA among children. There is an urgent need for such guidelines based on the existing evidence from developing countries.

DELAYED DIAGNOSIS OF DKA

What predisposes children with DKA to such complications in developing countries needs to be addressed urgently. Delayed diagnosis in DKA has been identified to be one of the factors for mortality in DKA in studies from Chennai-India[3] and also recently has been presented as an e poster at a conference, from Chandigarh-India[41]. Children with diabetes presenting with DKA at the onset has been attributed to delay in diagnosis in developed countries. Missed diagnosis of DKA predisposing the child to DKA is common in literature. However delay in diagnosis as a significant risk factor for mortality in DKA has been identified only from India[3]. This study reported that children with DKA had 1-5 physician visits prior to diagnosis of DKA. Children with DKA were more likely to have consulted a physician prior to diagnosis of DKA as reported in literature from developed countries. Rosenbloom[39] from US had mentioned that children with new onset DKA has been seen in physician's office prior to diagnosis without adequate history and laboratory evaluation. In infants and young children symptoms may be nonspecific and this needs a high index of suspicion to diagnose DKA. Literature reports that DKA has been misdiagnosed as surgical emergencies with acute abdomen[42]. Bui *et al*[43] from Canada published that among 285 children with DKA, 38.8% and 1104 children with diabetes with no DKA, 34.4% had at least one medical visit during the week before diagnosis (p-026). Ali *et al*[44] had published in 2011 that 30% of newly diagnosed children have had at least one related medical visit prior to diagnosis, suggesting the condition is being missed by doctors. Majaliwa *et al*[45] from Africa mention in their article that DKA can easily be misdiagnosed as cerebral malaria or meningitis in busy emergency reception areas of most hospitals in Africa. Literature reveals similar studies from Tunisia and Tanzania[46,47]. However none of these studies have identified delayed diagnosis as a risk factor for mortality in children with DKA. Study from Chen-

Table 1 Reasons for delayed diagnosis in diabetic keto acidosis

Reason
Lack of parental awareness about diabetic symptoms
Lack of awareness among physicians
Mis-interpretation of diabetic symptoms
Exclusive treatment of intercurrent illness only
Lack of finger prick estimation of blood glucose
Non recognition of lab abnormalities
Lack of immediate referral
Delay in transport to appropriate center
Improper referral
Due to parental causes (native treatment, social reasons and economic constraints)

nai[3] has identified delayed diagnosis in DKA in 64.8% of children with new onset DKA. Eighty-four point seven percent of infants and 58% of school children with DKA had delayed diagnosis. Delayed diagnosis was encountered in 12 of 13 children who died of DKA in their study. Specific risk factor related mortality for delayed diagnosis was 21% in the study[3]. Factors identified for the delayed diagnosis in their study is summarized in Table 1. Tachypnea in DKA has been universally misdiagnosed as bronchopneumonia, bronchiolitis or acute severe asthma in children. Polyuria and polydipsia have been misinterpreted as UTI in most of the children. Abdominal pain is misinterpreted as worm infestation and acute gastritis. Dehydration in the presence of vomiting is usually treated as acute gastroenteritis. Recent onset bed wetting is seen as a sign of stress in school children. Literature reveals up to 15%-86% of children with DKA not been diagnosed as diabetic at the first physician consultation[43,44,48,49].

Delay due to missed diagnosis is universal in DKA among children from developed and developing countries. Literature also reveals that, simple estimation of blood glucose by capillary method using finger prick has been used very rarely in physician consultation room[3]. A simple investigation in the physician's consultation room could have led to the diagnosis in children who had their diagnosis missed prior to DKA. Similarly, laboratories did not alert the treating physician or the parent when they measured high blood glucose or documented glycosuria in children[3]. Inappropriate referral was again reason for delay in specific management as the facilities for management of DKA by a structured diabetic care team or intensive care pediatrician is not universally available in developing countries. All treating physicians should have access to the standard treatment protocols for management of children with DKA or should have an access to help through hot line facilities at times of need. These factors coupled with lack of knowledge about emergency free public transport facilities, economic constraints and unhealthy cultural practices lead to delay in management of DKA in children from developing countries.

CONCLUSION

Analyzing the magnitude of problems in DKA in children from developing countries it is obviously evident that the mortality rates and reasons for such high mortality in DKA to be very different from developed countries. However majority of standard treatment protocols followed in developing countries are based on recommendation from developed countries. Root cause for majority of these complications could be delayed diagnosis of DKA. There is an urgent need for modified protocols for children with DKA and shock or renal failure. Fluid trials in such children is an urgent need of the hour. There exists difficulty in recognizing the symptoms of diabetes among parents and physicians[50]. With regard to delay in diagnosis there is a need for creating awareness among parents and physicians regarding clinical features of DM and DKA. The best strategy would be to identify DKA early and refer them to appropriate centers for management immediately. The laboratories should raise high risk alert immediately to the parent or the physician when they encounter a child's report with hyperglycemia and/or glycosuria.

As majority of the risk factors identified for mortality in DKA among children from developing countries are pretreatment factors, the ultimate aim of future programmes should be to prevent DKA in children. DKA occurring in new onset DM or in a known diabetic child is considered as a preventable health care failure. Vanelli *et al*[51,52] in Parma, Italy have proved that simple awareness programmes in schools and physicians office in the form of posters depicting signs of diabetes have helped over 5 years to reduce occurrence of DKA to zero. This has been proved to be successful even years after the programme was stopped. Studies from Australia have shown reduction in the rate of DKA at initial diagnosis of diabetes, during awareness campaigns[53]. Similar models with modification to local needs can help prevent delay in diagnosis of DKA among children from developing countries. Initiatives and awareness programmes need to be implemented in countries like India where the magnitude of the problem is likely to increase over years. It is time that emergency interventions are undertaken to minimize deaths in DKA in developing countries. Increased awareness among parents, school teachers and physicians is urgently warranted for early diagnosis and prevention of mortality in DKA. Creating awareness through nationwide diabetic awareness day can help an earlier diagnosis of DKA among children.

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ORIGINAL ARTICLE

Type 2 diabetes is associated with a worse functional outcome of ischemic stroke

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Abstract

AIM: To assess whether ischemic stroke severity and outcome is more adverse in patients with type 2 diabetes mellitus (T2DM).

METHODS: Consecutive patients hospitalized for acute ischemic stroke between September 2010 and June 2013 were studied prospectively (n = 482; 40.2% males, age 78.8 ± 6.7 years). T2DM was defined as self-reported T2DM or antidiabetic treatment. Stroke severity was evaluated with the National Institutes of Health Stroke Scale (NIHSS) score at admission. The outcome was assessed with the modified Rankin scale (mRS) score at discharge and with in-hospital mortality. Adverse outcome was defined as mRS score at discharge ⩾ 2 or in-hospital death. The length of hospitalization was also recorded.

RESULTS: T2DM was present in 32.2% of the study population. Patients with T2DM had a larger waist circumference, higher serum triglyceride and glucose levels and lower serum high-density lipoprotein cholesterol levels as well as higher prevalence of hypertension, coronary heart disease and congestive heart failure than patients without T2DM. On the other hand, diabetic patients had lower low-density lipoprotein cholesterol levels and reported smaller consumption of alcohol than non-diabetic patients. At admission, the NIHSS score did not differ between patients with and without T2DM (8.7 ± 8.8 and 8.6 ± 9.2, respectively; P = NS). At discharge, the mRS score also did not differ between the two groups (2.7 ± 2.1 and 2.7 ± 2.2 in patients with and without T2DM, respectively; P = NS). Rates of adverse outcome were also similar in patients with and without T2DM (62.3% and 58.5%, respectively; P = NS). However, when we adjusted for the differences between patients with T2DM and those without T2DM in cardiovascular risk factors, T2DM was independently associated with adverse outcome [relative risk (RR) = 2.39; 95%CI: 1.21-4.72, P = 0.012]. In-hospital mortality rates did not differ between patients with T2DM and those without T2DM (9.0% and 9.8%, respectively; P = NS). In multivariate analysis adjusting for the difference in cardiovascular risk factors between the two groups, T2DM was again not associated with in-hospital death.

CONCLUSION: T2DM does not appear to affect ischemic stroke severity but is independently associated with a worse functional outcome at discharge.

Key words: Ischemic stroke; Functional outcome; Severity; Mortality; Type 2 diabetes mellitus; Cardiovascular disease; Hyperglycemia; Cardiovascular risk; Dyslipidemia; Hypertension

Core tip: Even though type 2 diabetes mellitus (T2DM) is a major independent risk factor for ischemic stroke, it is unclear whether stroke severity and functional outcome differs between diabetic and non-diabetic patients. In the present study, T2DM was associated with worse functional outcome at discharge despite similar stroke severity at admission. The detrimental effect of T2DM on functional outcome was independent of the increased prevalence of cardiovascular risk factors in diabetic patients.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a major independent risk factor for cardiovascular disease (CVD), including stroke[1]. In a meta-analysis of 102 prospective studies (n = 698782), patients with T2DM had 2.27 times higher risk for ischemic stroke[1]. Moreover, in the INTERSTROKE study, a case-control study in 22 countries worldwide, T2DM accounted for 5% of the population-attributable risk for stroke[2]. Given the rising prevalence of T2DM due to the epidemic of obesity, the number of patients suffering stroke due to T2DM is expected to further increase[3,4].

In contrast to the unequivocal association between T2DM and the increased risk for ischemic stroke, it is unclear whether patients with T2DM suffer more severe strokes or have worse outcome following stroke compared with subjects without T2DM[5-7]. Moreover, it is uncertain whether T2DM is independently associated with more severe stroke and with worse stroke outcome or if this relationship is due to the higher prevalence of other CVD risk factors in patients with T2DM, including hypertension, dyslipidemia and obesity[5-7].

The aim of the present study was to evaluate the association between T2DM and acute ischemic stroke severity and in-hospital outcome. Furthermore, we aimed to examine whether T2DM affects stroke severity and outcome independently from other CVD risk factors.

MATERIALS AND METHODS

We prospectively studied all patients who were admitted to our department with acute ischemic stroke between September 2010 and June 2013 (n = 482; 40.2% males, age 78.8 ± 6.7 years).

At admission, demographic data (age, sex), history of T2DM and other cardiovascular risk factors [hypertension, atrial fibrillation, smoking, alcohol consumption, family history of CVD, chronic kidney disease], history of concomitant CVD [coronary heart disease (CHD), previous stroke, congestive heart failure] and pharmacological treatment were recorded. T2DM was defined as self-reported T2DM or antidiabetic treatment. Anthropometric parameters (weight, height, waist and hip circumference, waist to hip ratio) and systolic and diastolic blood pressure were also measured. The severity of stroke was assessed at admission with the National Institutes of Health Stroke Scale (NIHSS) score.

Routine laboratory investigations were performed after overnight fasting on the first day after admission and included serum levels of glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), creatinine, uric acid and HbA_{1c}. Low-density lipoprotein cholesterol (LDL-C) levels were calculated using Friedewald's formula[8]. Glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease equation[9]. Chronic kidney disease was defined as estimated GFR < 60 mL/min per 1.73 m^2.

All patients underwent brain computed tomography at admission and a second brain computed tomography was performed if clinically indicated.

All patients without atrial fibrillation were treated with aspirin; clopidogrel was given to patients intolerant to aspirin. Patients who were on aspirin prior to stroke were switched to clopidogrel and *vice versa*. Patients with atrial fibrillation were treated with low-molecular weight heparin. All patients were given a statin. Antihypertensive agents were discontinued during the acute phase of stroke except beta-blockers. Most patients with T2DM were treated with insulin during the acute phase of stroke. No patient underwent thrombolysis.

The outcome was assessed with the modified Rankin scale (mRS) score at discharge and with in-hospital mortality. Adverse outcome was defined as mRS score at discharge ⩾ 2 or in-hospital death. The length of hospitalization was also recorded.

Statistical analysis

All data were analyzed with the statistical package SPSS (version 17.0; SPSS, Chicago, IL, United States). Data are presented as percentages for categorical variables and as mean and standard deviation for continuous variables. Differences in categorical and continuous variables between groups were assessed with the χ^2 test and one-way analysis of variance, respectively. Binary logistic regression analysis was performed to evaluate the independent association between T2DM and adverse outcome or in-hospital mortality after adjusting for the differences in CVD risk factors between patients with and without T2DM. In all cases, a two-tailed $P < 0.05$ was considered significant.

RESULTS

T2DM was present in 32.2% of the study population.

Table 1 Clinical characteristics of patients with type 2 diabetes mellitus and those without

	Patients with T2DM (n = 155)	Patients without T2DM (n = 327)	P
Age (yr)	78.3 ± 6.3	79.1 ± 6.9	NS
Males (%)	38.7	41.0	NS
Systolic blood pressure (mmHg)	150 ± 24	146 ± 25	NS
Diastolic blood pressure (mmHg)	81 ± 10	81 ± 14	NS
Hypertension (%)	88.4	78.6	0.013
Smoking (current/past, %)	12.9/22.6	11.6/20.5	NS
Package-years	17 ± 39	14 ± 33	NS
Atrial fibrillation (%)	38.7	34.6	NS
Alcohol consumption (units/wk)	0.7 ± 2.4	2.1 ± 11.7	0.045
Weight (kg)	77.0 ± 13.0	73.7 ± 13.8	0.04
Body mass index (kg/m^2)	28.1 ± 5.2	27.2 ± 5.1	NS
Waist (cm)	110 ± 9	101 ± 13	< 0.001
Waist/hip	1.00 ± 0.06	0.97 ± 0.08	NS
Overweight/obese (%)	44.1/26.9	38.5/25.0	NS
Family history of cardiovascular disease (%)	14.8	15.0	NS
Coronary heart disease (%)	35.5	23.9	0.01
Previous ischemic stroke (%)	44.5	37.9	NS
Chronic kidney disease (%)	36.9	33.5	NS
Chronic heart failure (%)	26.5	16.5	0.015

T2DM: Type 2 diabetes mellitus; NS: Not significant.

The mean duration of T2DM was 11.1 ± 8.2 years and the mean HbA_{1c} in patients with T2DM was 7.6 ± 1.5. Clinical characteristics of patients with T2DM and patients without T2DM are shown in Table 1. Patients with T2DM had larger waist circumference and higher prevalence of hypertension, CHD and congestive heart failure than patients without T2DM but reported a lower consumption of alcohol than the latter. Laboratory characteristics of patients with T2DM and patients without T2DM are shown in Table 2. Patients with T2DM had higher serum TG levels and lower serum HDL-C levels than patients without T2DM but had lower LDL-C levels than the latter ($P < 0.01$ for all comparisons). Serum glucose levels were also higher in the former.

At admission, the NIHSS score did not differ between patients with and without T2DM (8.7 ± 8.8 and 8.6 ± 9.2, respectively; P = NS). The outcome of the 2 groups is shown in Table 3. The duration of hospitalization was comparable in patients with and without T2DM (6.9 ± 4.6 d and 6.7 ± 4.1 d, respectively; P = NS). The mRS score at discharge also did not differ between the two groups (2.7 ± 2.1 and 2.7 ± 2.2 in patients with and without T2DM, respectively; P = NS). The NIHSS score at discharge was also comparable in patients with and without T2DM (6.2 ± 6.4 and 6.0 ± 6.2, respectively; P = NS). Rates of adverse outcome were also similar in patients with and without T2DM (62.3% and 58.5%, respectively; P = NS). However, when we adjusted for the differences between patients with T2DM and those without T2DM in cardiovascular risk factors (weight, consumption of alcohol, prevalence of hypertension, CHD and congestive heart failure, and serum LDL-C, TG and HDL-C levels), T2DM was independently associated with adverse outcome [relative risk (RR) = 2.39; 95%CI: 1.21-4.72, P = 0.012]. In-hospital mortality rates did not differ between patients with T2DM and those without T2DM (9.0% and 9.8%, respectively; P = NS). In multivariate analysis adjusting for the difference in cardiovascular risk factors between the two groups, T2DM was again not associated with in-hospital death.

We also evaluated whether T2DM duration and glycemic control were associated with stroke severity and outcome. At admission, the NIHSS score did not differ between patients with T2DM duration > 10 years (n = 64, 41.3% of patients with T2DM), patients with T2DM duration ≤ 10 years and patients without T2DM (8.8 ± 9.0, 7.7 ± 8.2 and 8.6 ± 9.2, respectively; P = NS) or between patients with T2DM and HbA_{1c} > 9% (n = 28, 18.1% of patients with T2DM), patients with T2DM and HbA_{1c} ≤ 9% and patients without T2DM (8.4 ± 9.8, 10.6 ± 9.5 and 8.6 ± 9.2, respectively; P = NS). In univariate analysis, the duration of hospitalization, the mRS score at discharge and the rates of adverse outcome at discharge did not differ between patients with T2DM duration > 10 years, patients with T2DM duration ≤ 10 years and patients without T2DM. In multivariate analysis, both patients with T2DM duration > 10 years and patients with T2DM duration ≤ 10 years had higher risk for adverse outcome than patients without T2DM (RR = 2.66; 95%CI: 1.17-6.08 and RR = 2.60; 95%CI: 1.05-7.49, respectively; P = 0.030). The risk for adverse outcome did not differ between patients with T2DM duration > 10 years and patients with T2DM duration ≤ 10 years. In contrast, in-hospital mortality rates did not differ between patients with T2DM duration > 10 years, patients with T2DM duration ≤ 10 years and patients without T2DM in either univariate or multivariate analysis. The duration of hospitalization, the mRS score at discharge and the rates of adverse outcome at discharge and in-hospital mortality also did not differ between patients with T2DM and HbA_{1c} > 9%, patients with T2DM and HbA_{1c} ≤ 9% and patients without T2DM in either univariate or multi-

Table 2 Laboratory characteristics of patients with type 2 diabetes mellitus and those without

	Patients with T2DM (n = 155)	Patients without T2DM (n = 327)	P
Glucose (mg/dL)	145 ± 64	99 ± 27	< 0.001
LDL-C (mg/dL)	103 ± 43	116 ± 38	0.007
HDL-C (mg/dL)	43 ± 14	48 ± 15	0.002
Triglycerides (mg/dL)	136 ± 63	112 ± 44	0.001
Uric acid (mg/dL)	5.8 ± 1.8	5.7 ± 1.9	NS
eGFR (mL/min per 1.73 m^2)	67 ± 22	70 ± 23	NS

T2DM: Type 2 diabetes mellitus; NS: Not significant; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; eGFR: Estimated glomerular filtration rate.

Table 3 Severity of stroke and outcome of patients with type 2 diabetes mellitus and those without

	Patients with T2DM (n = 155)	Patients without T2DM (n = 327)	P
National Institutes of Health Stroke Scale score at admission	8.7 ± 8.8	8.6 ± 9.2	NS
Duration of hospitalization (d)	6.9 ± 4.6	6.7 ± 4.1	NS
Modified Rankin scale score at discharge	2.7 ± 2.1	2.7 ± 2.2	NS
Adverse outcome (%)	62.3	58.5	NS
In-hospital mortality (%)	9	9.8	NS

T2DM: Type 2 diabetes mellitus; NS: Not significant.

variate analysis.

DISCUSSION

The main findings of the present study are that the severity of ischemic stroke does not appear to differ between patients with T2DM and those without T2DM. In contrast, T2DM independently portends a more adverse functional outcome at discharge in this population.

The neurological deficit at admission, evaluated with the NIHSS, was almost identical in diabetic and non-diabetic patients in our study (8.7 ± 8.8 and 8.6 ± 9.2, respectively; P = NS). A few studies have compared stroke severity between patients with T2DM and without T2DM, yielding conflicting results[10-13]. The two largest studies (n = 233 and 611 patients with T2DM) reported no association between T2DM and stroke severity, in accordance with our findings[10,11]. In contrast, an early small study (n = 50 diabetic patients) suggested that stroke is more severe in patients with T2DM; however, stroke severity was evaluated with a non-validated neurological index[12]. Finally, in a more recent report (n = 102 diabetic patients), patients with T2DM had a less severe stroke at admission[13]. The latter study included younger patients and a higher percentage of males than the present study; it is possible that this might have contributed to the less severe stroke presentation in diabetic patients since T2DM appears to increase CVD risk more in women and in older subjects[1]. Indeed, among patients with T2DM who suffer an ischemic stroke, women have a less favorable prognosis than men[14]. Nevertheless, the discordant findings regarding the association between T2DM and ischemic stroke severity stress the need for larger studies to resolve these discrepancies.

Even though the rates of adverse outcome at discharge did not differ between diabetic and non-diabetic patients in unadjusted analyses in our study, binary logistic regression analysis adjusting for differences in CVD risk factors between the 2 groups identified an independent association between T2DM and adverse outcome (RR = 2.39). Therefore, our findings suggest that T2DM has a detrimental effect on ischemic stroke and that this association is not fully explained by the increased prevalence of other CVD risk factors in diabetic patients. Indeed, several studies suggested that hyperglycemia per se predicts worse outcomes in patients with ischemic stroke[15]. On the other hand, administration of insulin to maintain normoglycemia in these patients does not appear to improve functional outcome or to reduce in-hospital mortality[16,17]. Moreover, there is a paucity of studies that assessed the relationship between T2DM and functional outcome at discharge in acute ischemic stroke[13,18-20]. Both studies that adjusted for confounding variables reported a worse outcome in diabetic patients[18,19], whereas both studies that reported only unadjusted analyses did not identify any difference in functional outcome between patients with T2DM and patients without T2DM[13,20]. Accordingly, more studies are needed to evaluate whether T2DM affects functional outcome and to clarify the pathogenetic mechanisms underpinning this association.

In-hospital mortality rates did not differ between diabetic and non-diabetic patients in our study. This lack of difference was observed both in unadjusted analyses and when we adjusted for confounding variables. Some previous studies with only unadjusted analyses reported similar findings[13,20], whereas in-hospital mortality was higher in diabetic patients in a recent large study when multivariate analysis was performed[19]. Notably, in the latter study, mortality rates were identical in patients with and without T2DM in univariate analyses. Therefore, it is possible that

our study lacked the statistical power to detect a difference in mortality rates between diabetic and non-diabetic patients because of the low case-fatality rate.

Our study has some limitations. Although diabetic patients had poorer short-term functional prognosis in our population, previous studies showed that the subgroup of diabetic patients with lacunar infarction shows a better outcome[21,22]. However, magnetic resonance imaging is not available in our institution and imaging of the intra- or extracranial arteries was also not performed in all patients. Therefore, we cannot determine the frequency of the different stroke subtypes in our population. Moreover, the location of stroke, which may influence the functional outcome, was not systematically recorded. Finally, since we did not evaluate urinary albumin excretion in all patients, we were not able to evaluate the effects of albuminuria on stroke severity or outcome.

In conclusion, T2DM does not appear to affect ischemic stroke severity but is associated with worse functional outcome at discharge. This detrimental effect of T2DM on short-term stroke outcome appears to be independent of the increased prevalence of CVD risk factors in diabetic patients. Accordingly, management of hyperglycemia might have beneficial effects in patients with acute ischemic stroke but this remains to be established in prospective controlled trials.

COMMENTS

Background

Type 2 diabetes mellitus (T2DM) is a major independent risk factor for cardiovascular disease, including ischemic stroke. However, it is unclear whether stroke is more severe in patients with T2DM than in non-diabetic patients and whether the outcome of ischemic stroke is less favorable in the former.

Research frontiers

There is active research in the field of the potential role of strict glycemic control in the management of diabetic patients during the acute phase of ischemic stroke. Therefore, it is important to clarify whether hyperglycemia *per se* exerts a detrimental effect on stroke outcome.

Innovations and breakthroughs

The present study suggests that patients with T2DM have a less favorable functional outcome than non-diabetic patients even though stroke severity does not differ between the two groups. The adverse effect of T2DM on stroke outcome also appears to be independent of the increased prevalence of other cardiovascular risk factors in patients with T2DM (*e.g.*, hypertension, dyslipidemia, obesity) and appears to be mediated by hyperglycemia *per se*.

Applications

Since hyperglycemia appears to be associated with worse functional outcome in diabetic patients admitted with acute ischemic stroke, it is possible that strict glucose control might improve the outcome of this population. However, randomized controlled trials are needed to validate this hypothesis.

Terminology

In the present study, stroke severity was evaluated with the National Institutes of Health Stroke Scale, a comprehensive index of neurological deficit; a higher NIHSS signifies a more severe stroke. Functional outcome was assessed with the modified Rankin Scale (mRS), which quantifies disability and ranges from lack of symptoms (mRS = 0), no disability despite symptoms (mRS = 1), to progressively more severe disability (mRS = 2-5) and death (mRS = 6); adverse outcome was defined as mRS $\geqslant$ 2, *i.e.*, dependency or death.

Peer review

The study is important especially when from different populations.

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OBSERVATIONAL STUDY

Risk factors for cost-related medication non-adherence among older patients with diabetes

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Abstract

AIM: To assess the risk factors for cost-related medication non-adherence (CRN) among older patients with diabetes in the United States.

METHODS: We used data from the 2010 Health and Retirement Study to assess risk factors for CRN including age, drug insurance coverage, nursing home residence, functional limitations, and frequency of hospitalization. CRN was self-reported. We conducted multivariate regression analysis to assess the effect of each risk factor.

RESULTS: Eight hundred and seventy-five (18%) of 4880 diabetes patients reported CRN. Age less than 65 years, lack of drug insurance coverage, and frequent hospitalization significantly increased risk for CRN. Limitation in both activities of daily living and instrumental activities of daily living were also generally associated with increased risk of CRN. Residence in a nursing home and Medicaid coverage significantly reduced risk.

CONCLUSION: These results suggest that expanding prescription coverage to uninsured, sicker, and community-dwelling individuals is likely to produce the largest decreases in CRN.

Key words: Cost; Medication; Non-adherence; Risk factors

Core tip: Using a nationally representative date set, this study explores a wide range of risk factors influencing cost-related medication non-adherence (CRN), which receives increasing recognition of importance in diabetes. The authors found that age less than 65, lack of prescription drug insurance coverage, increased numbers of hospitalizations, and greater functional limitations were associated with higher likelihood of CRN among diabetic patients, while nursing home residence decreased risk. Together, these results suggest that expanding prescription coverage to uninsured, sicker, and community-dwelling individuals is likely to produce the largest decreases in CRN.

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INTRODUCTION

Up to a third of older patients report cost-related medication non-adherence (CRN)[1]. Lower income and high out-of-pocket costs for medications, poorer health status including lower self-perceived general health, more comorbidities, and poorer mental health, are strong risk factors for CRN, while having any, or more generous, prescription drug coverage significantly reduces the risk of

CRN[2-6]. Increased costs of prescription drugs are associated with lower rates of medication use, poor health outcomes, more hospitalizations, and increased use of medical services, including emergency department visits[7-9].

There is an increasing recognition of the importance of CRN in diabetes. Diabetic patients often require a large number of prescription drugs and incur high out-of-pocket costs for medications and medical expenses[10,11]. There is an emerging body of studies examining CRN for diabetes patients, reporting CRN rates ranging from 14% to 30% depending on the study sample[12-17]. However, little is known about the factors associated with CRN in diabetes patients, particularly those who have not yet reached 65 years of age (when they typically become eligible for Medicare), reside in a nursing home, have had multiple hospitalizations, or who have functional limitations. In these patients, medication non-adherence can significantly reduce the effectiveness of care, place them at an increased risk of declining health, and incur significant downstream costs. In addition, several of these risk factors can be potentially modified through social policy and clinical practice. Our aim was to assess variation in CRN with a broad set of risk factors for diabetes patients over the age of 50 using a nationally representative dataset.

MATERIALS AND METHODS

Study population

We utilized the 2010 data from the Health and Retirement Study (HRS). The HRS is an ongoing longitudinal cross-sectional study that surveys a nationally representative sample of Americans over the age of 50 about their income, employment, health insurance, physical health, cognitive functioning, and health care expenditures[18]. Data for the survey is collected primarily by telephone interview every 2 years. The analysis in this study was restricted to survey respondents who reported that a physician had told them that they had diabetes.

CRN

CRN was measured by asking participants, "Sometimes people delay taking medication or filling prescriptions because of the cost. At any time since the last interview or in the last two years have you ended up taking less medication than was prescribed for you because of the cost?" Participants answered either yes or no, although they had the option to refuse to answer or say that they did not know.

Demographic and socio-economic characteristics

The HRS includes questions about demographics and socio-economic characteristics, including age, place of birth, education level, ethnicity, employment, and place of residence. We categorized patients into age groups of 50-64 years, 65-74 years, 75-84 years, and 85 years and older. We hypothesized that patients in the age group of 50-64 years old might be at elevated risk of CRN because they may not have had adequate protection from employer-sponsored health insurance and were too young to be eligible for Medicare which could provide low-cost outpatient drug insurance benefits. In addition, depending on the patient's current health status, it may have been difficult to purchase individual health insurance due to pre-existing conditions.

We included a variable indicating residence in a nursing home. We hypothesized that living in a nursing home and administration of medications by the nursing staff would decrease the risk of CRN. In addition, nursing home patients were more likely to qualify for Medicaid due to low income, and thus out-of-pocket payments for medications should also be reduced, subsequently decreasing the risk of CRN further.

We also included a variable indicating whether the costs of prescription medications were covered at all by health insurance, which may be especially important for low-income persons. We also included a variable indicating patients' insurance coverage by Medicaid, as Medicaid coverage for the poor may enable their ability to purchase needed drugs.

To describe the resultant burden of out-of-pocket payments for medications with or without medication insurance coverage, we calculated average monthly out-of-pocket expenses for medications, based upon responses to the HRS survey question, "On average, about how much have you paid out-of-pocket per month for these prescriptions since last interview/in the last 2 years?" If they did not know, the interviewer would ask whether it amounted to less or more than a certain dollar amount.

Functional status and number of hospitalizations

HRS asks participants about functional status through questions on limitations in activities of daily living (ADLs)[19] and instrumental activities of daily living (IADLs)[20], with higher numbers of limitations indicating worse functional status. Functional limitations may reflect the effects of underlying diseases such as advanced diabetes or other chronic diseases, and can act as barriers to purchasing and administering medications as prescribed.

The HRS also collects information about healthcare utilization, including hospitalizations and physician visits. Participants were asked the number of different times they were hospitalized overnight in the past two years, as well as how many nights they stayed. We hypothesized that while hospitalizations result in out-of-pocket payments that could affect patients' ability to pay for medications, such effects on CRN might be small given Medicare's generous coverage for hospitalizations.

We also included an indicator variable for the class of prescription medications each respondent reported taking, including medications for cholesterol, joint or muscle pain, asthma or allergies, stomach problems, insomnia, and anxiety or depression.

Statistical analysis

We first performed bivariate analyses of the association

between CRN and socio-demographic variables, limitations in ADLs and IADLs, number of hospitalizations, medication insurance coverage, and self-reported monthly out-of-pocket (OOP) payments of prescription drugs. We examined differences in CRN for varying levels of limitations in ADLs and IADLs, medication insurance coverage and socio-demographic variables by utilizing χ^2 statistics. To evaluate differences in OOP payments for prescription drugs for those with and without CRN, we performed *t*-tests. We then analyzed the association between the number of hospitalizations and CRN by using a general linear regression model, using those without any hospitalizations as the reference group.

We further conducted multivariate regression analysis to assess the net effect of the aforementioned risk factors on CRN. In this case, a logit model was used to assess the independent risk factors including age, nursing home residence, medication insurance coverage, varying level of limitations in ADLs and IADLs, hospitalizations, and medication use for common conditions.

RESULTS

Among 22042 respondents in the 2010 HRS, 5037 (23%) reported that they were told by a physician that they had diabetes. The mean age of the 5037 diabetes patients was 67 years (s.d. 11). One-hundred fifty-seven patients (3.1%) were younger than 50 years old and were subsequently excluded from the analysis, resulting in a final sample of 4880 adults. Among the 4880 diabetes patients in the final sample, 875 patients, or 18.3%, reported CRN in the past 2 years.

Of the 875 patients who reported CRN, 573 (65.5%) were between the ages of 50 and 64 years old. Table 1 shows the prevalence of CRN by different socio-demographic variables. Females, African-Americans and Hispanics were more likely to report CRN. As expected, those without any insurance coverage for medications were significantly more likely to report CRN than those with coverage (38% *vs* 16%, $P < 0.001$). There also appeared to be differences in CRN in survey respondents reporting no functional limitations compared to those with 1 or more limitations in ADLs or IADLs, with those with functional limitations more likely to report CRN (25% *vs* 15% for ADLs, $P < 0.001$; 23% *vs* 16% for IADLs, $P < 0.001$). Respondents with at least 1 overnight hospitalization in the past 2 years were also significantly more likely to report CRN compared to those who were never hospitalized (22% *vs* 16%, $P < 0.001$). Nursing home residents had a much lower rate of CRN than community dwellers (5% *vs* 18%, $P < 0.001$). Diabetes patients covered by Medicaid were significantly less likely to report CRN ($P < 0.001$). Respondents who reported CRN had higher monthly out-of-pocket payments for prescription drugs ($P < 0.001$).

Table 2 shows the independent risk factors of CRN in the multivariate logistic regression model. Compared to respondents who were in the 65-74 years age group, those in the age group of 50-64 years were 118% more likely to report CRN. The likelihood of CRN also decreased as patient age advanced. Patients residing in nursing home were 66% less likely to report CRN compared to patients living in the community, and patients without drug insurance coverage were 182% more likely to report CRN compared to those with drug insurance coverage. Patients covered by Medicaid were 66% less likely to report CRN.

Compared to those without any limitations, survey respondents with 1 or more limitations in ADLs or IADLs were much more likely to report CRN, although confidence intervals were wide for the categories with the highest number of limitations so that having 6 or more limitations in ADLs or 3 or more limitations in IADLs were not statistically significant.

Compared to those without any hospitalizations, having any number of hospitalizations increased the risk of CRN. The magnitude of effect on the risk of CRN increased as the number of hospitalizations increased, with a slight decrease for those with 4 or more hospitalizations.

While the coefficients reflecting the effect of each class of medications were all positive in the multivariate logistic regression, in general, they were not statistically significant with the exception of asthma ($P = 0.01$).

DISCUSSION

We found that diabetes patients ages 50-64 years old were at increased risk for CRN. A recent report suggests that this age group is at increased risk of being uninsured despite being employed, and due to higher insurance premiums based upon their age and health, it is more difficult for individuals to obtain health insurance elsewhere[21]. That CRN is increased in this age group in our multivariate analysis, which controls for insurance status, suggests that there are other factors besides medication insurance coverage contributing to the higher risk. One factor could be the level of out-of-pocket payments. Although the Affordable Care Act will expand Medicaid eligibility for poor individuals and families, and coverage cannot be denied based on pre-existing conditions, these findings suggest the importance of insurance benefit design so that high-value treatments in diabetes care can be obtained with low out-of-pocket payments. In addition, it is also possible that pent-up demand may be another source of delay in seeking medical care as patients approach the eligibility age of Medicare. Further researches are needed to understand the patient behavior in this aspect.

Our study also found that living in a nursing home is protective for CRN. The high rate of Medicaid coverage among nursing home residents could explain this finding in part as the out-of-pocket payments to medication are nominal for Medicaid beneficiaries. In addition, the administration of medication by the nursing home staff may also reduce the costs of obtaining the medication such as travel, time, and mobility. Overall, Medicaid coverage significantly reduces CRN.

Table 1 Prevalence of cost-related medication non-adherence by Socio-Demographics, Health Status and Usage of Medical Resources *n* (%)

	All	Reported CRN	Did not report CRN	*P* value
Full sample	4880 (100)	875 (18)	4005 (82)	
Age				
⩾ 50 and ⩽ 64	2031 (100)	573 (28)	1458 (72)	
⩾ 65 and ⩽ 74	1505 (100)	207 (14)	1298 (86)	
⩾ 75 and ⩽ 84	1017 (100)	82 (8)	935 (92)	< 0.001
⩾ 85	329 (100)	13 (4)	314 (96)	
Gender				
Male	2224 (100)	343 (15)	1881 (85)	
Female	2656 (100)	532 (20)	2124 (80)	< 0.001
Race				
White	3298 (100)	502 (15)	2796 (85)	
African-American	1187 (100)	277 (23)	910 (77)	
Other	393 (100)	94 (24)	299 (76)	< 0.001
Ethnicity				
Hispanic	883 (100)	189 (21)	694 (79)	
Non-Hispanic	3997 (100)	686 (17)	3311 (83)	0.003
Nursing Home				
Living in NH	138 (100)	7 (5)	131 (95)	
Not in NH	4742 (100)	868 (18)	3874 (82)	< 0.001
Insurance coverage for Rx				
Yes	4461 (100)	716 (16)	3745 (84)	
No	419 (100)	159 (38)	260 (62)	< 0.001
Medicaid coverage				
Currently covered	349 (100)	24 (7)	325 (93)	
Not covered	4531 (100)	851 (19)	3680 (81)	< 0.001
ADL limitations				
No limitation	3455 (100)	519 (15)	2936 (85)	
1 or more limitations	1425 (100)	356 (25)	1069 (75)	< 0.001
IADL limitations				
No limitation	3442 (100)	547 (16)	2895 (84)	
1 or more limitations	1438 (100)	328 (23)	1110 (77)	< 0.001
Hospitalization				
No hospitalization	3081 (100)	486 (16)	2595 (84)	
1 or more hospitalizations	1799 (100)	389 (22)	1410 (78)	< 0.001
Monthly out-of-pocket payments for Rx				
Payments: $ (s.d.)	69 (2.4)	108 (6.9)	60 (2.5)	< 0.001

P values by χ^2 tests, except for out-of-pocket payments for Rx, where *t*-test was performed. CRN: Cost-related medication non-adherence; ADL: Activities of daily living; IADL: Instrumental activities of daily living.

We found the female were more likely to report CRN. Previous researches have reported the gender-specific difference in non-adherence behaviors although the causes of non-adherence were not clear[22,23]. It was possible there was a difference in price-sensitivity to medication between the males and females. This highlighted the need for more researches in the gender difference in CRN in order to increase the adherence.

There was a positive association between CRN and limitations in ADLs and IADLs. This is concerning from a health perspective, since non-adherence may worsen their functional status and their medical disease further. However, the weak evidence for increased CRN among patients with extreme functional limitations in both ADLs and IADLs is notable and surprising. One answer might be that patients who are severely limited in their ability to take care of themselves tend to live in nursing homes and therefore likely to be covered by Medicaid, so that CRN is rare. However, we control for both these factors in our analysis. Another possibility is that the dependency of these individuals prompts others to provide them with assistance in obtaining medications that makes cost less of a barrier. However, this is purely speculative and this result seems worthy of further analysis.

The positive correlation between CRN and the number of hospitalizations is notable because non-adherence can potentially increase the likelihood of readmission, thus driving more expensive care. It is less clear from the data available whether the number of hospitalizations increases CRN, CRN increases hospitalization rates, or both. Future research should be directed at assessing the causal relationship between hospitalizations and CRN in such a high-risk patient population.

This study is limited in that while we have shown that a number of factors may be affecting CRN, we do not have measures of some key factors, such as insurance benefit design. As a result, the exact reason for CRN among those with drug insurance coverage is less clear. Also, the HRS survey does not give us any indication of how often participants did not take their medications due to cost, and only asks whether they had done so within the past 2 years.

Diabetes is a major chronic condition that causes significant mortality and morbidity, requiring coordina-

Table 2 Results of Multivariate Logistic Regression Analysis of Medication cost-related medication non-adherence

	OR	*P* value	95%CI
Age			
Age 50-64	2.182	< 0.001	1.805-2.638
Age 65-74	Reference	-	-
Age 75-84	0.532	< 0.001	0.403-0.704
Age ⩾ 85	0.236	< 0.001	0.131-0.426
Residence			
Nursing home residence	0.335	0.011	0.145-0.775
Medicare insurance coverage			
No drug coverage	2.824	< 0.001	2.233-3.570
Medicaid coverage			
Currently covered by Medicaid	0.341	< 0.001	0.217-0.535
Functional limitations			
Activities of daily living			
No limitation	Reference	-	-
1 limitation	1.431	0.005	1.113-1.840
2 limitations	1.850	< 0.001	1.348-2.540
3 limitations	1.813	0.003	1.229-2.674
4 limitations	1.796	0.012	1.137-2.838
5 limitations	1.776	0.023	1.083-2.911
6 limitations	1.669	0.162	0.813-3.425
Instrumental activities of daily living			
No limitation	Reference	-	-
1 limitation	1.376	0.006	1.094-1.731
2 limitations	1.494	0.014	1.084-2.060
3 limitations	1.243	0.307	0.819-1.887
4 limitations	0.872	0.651	0.482-1.579
Number of hospitalizations			
No hospitalization	Reference	-	-
1 hospitalization	1.320	0.010	1.068-1.632
2 hospitalizations	1.437	0.011	1.089-1.898
3 hospitalizations	1.683	0.005	1.168-2.424
4 or more hospitalizations	1.507	0.013	1.091-2.082
Medication use for common conditions			
Cholesterol	1.118	0.202	0.942-1.327
Pain	1.090	0.320	0.942-1.327
Asthma	1.295	0.011	1.062-1.578
Stomach	1.141	0.186	0.939-1.388
Sleep	1.077	0.516	0.861-1.347
Anxiety	1.082	0.453	0.880-1.330

Results from multivariate logistic regression analysis.

tion of care to treat the patient as a whole person[24]. It is a disease in which patient nonadherence to medications may be a result of a number of failures in social, economic, behavioral, and managerial aspects of care. Previous research suggests that insurance coverage alone does not guarantee high quality of diabetes care to patients[25], and more research is much needed to understand the influence of the hybrid of factors influencing CRN, in order to prevent the well-known complications of the disease that can debilitate patients further in the future.

In conclusion, despite the limitations of the study, the results imply that there are significant opportunities to reduce CRN and improve the effectiveness of pharmacotherapy in diabetes patients through public policy and clinical practice. More research is needed to elucidate the causal relationship between functional limitations, hospitalizations, and CRN. In addition, interventions that aim to reduce cost-cutting behaviors such as generic medication substitution in these patients have the potential of improving the effectiveness of treatment and reducing overall medical costs.

COMMENTS

Background

There is an increasing recognition of the importance of cost-related medication non-adherence (CRN) in diabetes. However, little is known about the factors associated with CRN in diabetes patients, particularly those who have not yet reached 65 years of age, reside in a nursing home, have had multiple hospitalizations, or who have functional limitations.

Research frontiers

Researches have shown that lower income and high out-of-pocket costs for medications, poorer health status including lower self-perceived general health, more comorbidities, and poorer mental health, are strong risk factors for CRN, while having any, or more generous, prescription drug coverage significantly reduces the risk of CRN. Increased costs of prescription drugs are associated with lower rates of medication use, poor health outcomes, more hospitalizations, and increased use of medical services, including emergency department visits

Innovations and breakthroughs

Using a nationally representative sample, the authors evaluated an array of risk factors of CRN including pre-Medicare (65 years old), residence status in nursing home, repeated hospitalizations, and functional limitations.

Applications

These results suggest that expanding prescription coverage to uninsured, sicker, and community-dwelling individuals is likely to produce the largest de-

creases in CRN.

Terminology

CRN: Cost-related medication non-adherence.

Peer review

The authors conclude that expanding prescription coverage to uninsured, sicker, and community-dwelling individuals is likely to produce the largest decreases in CRN. The findings are interesting.

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RANDOMIZED CONTROLLED TRIAL

Pancreas transplantation: The Wake Forest experience in the new millennium

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Abstract

AIM: To investigate the Wake Forest experience with pancreas transplantation in the new millennium with attention to surgical techniques and immunosuppression.

METHODS: A monocentric, retrospective review of outcomes in simultaneous kidney-pancreas transplant (SKPT) and solitary pancreas transplant (SPT) recipients was performed. All patients underwent pancreas transplantation as intent-to-treat with portal venous and enteric exocrine drainage and received depleting antibody induction; maintenance therapy included tapered steroids or early steroid elimination with mycophenolate and tacrolimus. Recipient selection was based on clinical judgment whether or not the patient exhibited measureable levels of C-peptide.

RESULTS: Over an 11.25 year period, 202 pancreas transplants were performed in 192 patients including 162 SKPTs and 40 SPTs. A total of 186 (92%) were primary and 16 (8%) pancreas retransplants; portal-enteric drainage was performed in 179 cases. A total of 39 pancreas transplants were performed in African American (AA) patients; of the 162 SKPTs, 30 were performed in patients with pretransplant C-peptide levels > 2.0 ng/mL. In addition, from 2005-2008, 46 SKPT patients were enrolled in a prospective study of single dose alemtuzumab *vs* 3-5 doses of rabbit anti-thymocyte globulin induction therapy. With a mean follow-up of 5.7 in SKPT *vs* 7.7 years in SPT recipients, overall patient (86% SKPT *vs* 87% SPT) and kidney (74% SKPT *vs* 80% SPT) graft survival rates as well as insulin-free rates (both 65%) were similar (P = NS). Although mortality rates were nearly identical in SKPT compared to SPT recipients, patterns and timing of death were different as no early mortality occurred in SPT recipients whereas the rates of mortality following SKPT were 4%, 9% and 12%, at 1-, 3- and 5-years follow-up, respectively ($P < 0.05$). The primary cause of graft loss in SKPT recipients was death with a functioning graft whereas the major cause of graft loss following SPT was acute and chronic rejection. The overall incidence of acute rejection was 29% in SKPT and 27.5% in SPT recipients (P = NS). Lower rates of acute rejection and major infection were evidenced in SKPT patients receiving alemtuzumab induction therapy. Comparable kidney and pancreas graft survival rates were observed in AA and non-AA recipients despite a higher prevalence of a "type 2 diabetes" phenotype in AA. Results comparable to those achieved in insulinopenic diabetics were found in the transplantation of type 2 diabetics with detectable C-peptide levels.

CONCLUSION: In the new millennium, acceptable

medium-term outcomes can be achieved in SKPT and SPTs as nearly 2/3rds of patients are insulin independent following pancreas transplantation.

Key words: Alemtuzumab; Mycophenolate mofetil; Pancreas transplantation; Portal-enteric; Rabbit anti-thymocyte globulin; Simultaneous kidney-pancreas transplantation; Solitary pancreas transplantation; Steroid elimination; Surveillance biopsy; Tacrolimus

Core tip: Vascularized pancreas transplantation is able to establish a chronic insulin-free state characterized by normoglycemia. In selected recipients with insulin-requiring diabetes, simultaneous kidney-pancreas transplantation has become acknowledged as a favored alternative to kidney alone transplantation because of more intense glucose control, enhanced quality of life and improved long-term survival. The evolution in surgical technique, current patient management strategies, and biopsy directed immunosuppression have resulted in excellent outcomes, even in populations previously considered high risk, such as African-American recipients, patients with a "type 2 diabetes" phenotype and solitary pancreas transplants recipients.

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INTRODUCTION

Although first developed as a modality to re-establish endogenous insulin secretion (C-peptide production) reactive to normal feedback controls, vascularized pancreas transplantation (PTx) has evolved over the past several years to complete β cell replacement that frees the patient both from the need to monitor serum glucose as well as the need to administer insulin in order to control diabetes. Patients who present following a total pancreatectomy for benign disease, or those with type 1 or type 2 diabetes, both of which require the administration of insulin, are appropriate candidates for PTx. In the search for a definitive treatment that restores normal glucose homeostasis in patients with complicated diabetes, and alleviates the risk of severe hypo/hyperglycemia, PTx is currently the only procedure that can accomplish this objective and may avert, stabilize, or reverse progressive diabetic complications.

As of December 2010, the International Pancreas Transplant Registry had received data on > 35000 PTxs whereas the Collaborative Transplant Study database had recorded nearly 9000 cases[1,2]. PTx in diabetic patients is separated into 3 chief categories; those performed following either a successful living or deceased donor kidney transplant [sequential pancreas after kidney (PAK) transplant], those occurring in patients with preserved native renal function [pancreas transplant alone (PTA)], and most commonly, those performed simultaneous with a kidney transplant (SKPT). The former 2 categories are frequently analyzed together as solitary pancreas transplants (SPT) because of similar outcomes. Until 2004, the annual number of PTxs progressively increased in the United States but has since declined, with particular reference to the PAK transplant category[1,3,4]. In the past 10 years, both the number of patients being added to the waiting list and the number of pancreata being recovered from deceased donors have decreased whereas the proportion of recovered pancreata being discarded and time on the waiting list for recipients have increased. In addition, recipient age and body mass index (BMI) have increased for PTx in the past decade concomitant with the proportion of recipients who are either African American (AA) or characterized as having type 2 diabetes[1,3,4].

At present, about 9% of PTxs are PTA, 16% PAK, and the remaining 75% are performed as SKPTs[1,3,4]. Success rates for PTx have progressively improved, secondary to refinements in diagnostic and therapeutic technologies and surgical techniques, advancements in immunosuppression and anti-infective prophylaxes, new and effective techniques in organ retrieval and preservation technology and increased experience in the selection of donors and recipients[1,3-5]. Over time, improvements in outcomes have occurred in all 3 PTx categories as a result of a decrease in technical failures and immunologic graft losses. At present, five-year patient survival rates are 89% in PTA, 87% in SKPT, and 83% in PAK transplant recipients. One-year patient survival is more than 95% in the cases of recipients of primary deceased donor PTxs whereas 10-year patient survival exceeds 70% in all 3 categories[1].

The definition of PTx graft survival is variable but principally defined as absolute freedom from exogenous insulin therapy, concomitant with the absence of atypical glycemic excursions, in contrast to other modalities utilized for the treatment of diabetes. According to Registry data, one-year insulin-free rates are currently 78% in PTA, 80% in PAK, and 85% in SKPT recipients. These data indicate that we may now expect pancreas graft half-lives approaching fourteen years in SKPT and ten years in SPT recipients[1,3-5]. The focus of this study was the retrospective review of PTx outcomes at our center in the emergent millennium.

MATERIALS AND METHODS

Recipient selection

Diabetes mellitus treated with exogenous insulin, the presence of diabetic complications, and the ability to endure the surgical procedure, were significant indications in the selection of candidates for PTx. In addition, there existed the need for these recipients to be predictably

able to manage the requisite immunosuppression and expected follow-up, irrespective of detectable C-peptide levels. The selection criteria for SKPT in type 2 diabetes have been previously reported[6-8]. Selection criteria for SPT were similar to SKPT except for renal function, in which the glomerular filtration rate (GFR), determined by the abbreviated Modification of Diet in Renal Diseases (aMDRD) formula, was > 70 mL/min in PTA (native renal function) and > 40 mL/min in PAK (renal allograft function) transplant recipients already on a calcineurin inhibitor. Donor selection was more stringent for SPT, including younger donors and a minimum of a 2-3 human leukocyte antigen (HLA) match[7,9].

Technical aspects

The history of PTx has been essentially defined by the evolving trends in surgical techniques. We performed our first SKPT at Wake Forest Baptist Health (WFBH) on 6/3/92[7]. The exocrine secretions were managed with bladder drainage using a short donor duodenal segment conduit. Although the patient initially did well with excellent dual allograft function, she ultimately required enteric conversion on 12/20/07 for persistent difficulties related to bladder drainage including dehydration, episodes of gross hematuria requiring blood transfusions, metabolic acidosis and recurrent urinary tract infections. At 22 years follow-up, this pancreas allograft continues to exhibit acceptable function and the patient remains insulin-free. The next PTx at WFBH was not performed until the latter part of 2001.

Since November, 2001, all PTxs were initially approached as intent-to-treat with portal-enteric drainage using an anterior approach to the superior mesenteric vein (SMV). Enteric drainage was performed by side to side duodeno-enterostomy to the recipient's proximal ileum[7,10]. We used diverting Roux limbs infrequently, which were reserved for cases in which the allograft duodenum did not reperfusion well. Arterial inflow was usually based on the recipient's right common iliac artery after the pancreas dual artery blood supply was reconstructed with a donor common iliac bifurcation "Y" graft. Relative "contraindications" to portal venous drainage have been previously reported[10]. In patients (particularly male) with a high BMI, the SMV can be quite deep in the mesentery and the donor common iliac artery bifurcation "Y" graft might not be long enough to reach the recipient's iliac artery through a window in the distal ileal mesentery, even with the liberal use of a donor artery "extension" graft. In these cases, systemic venous and enteric drainage were performed to simplify the procedure.

Of the first 121 SKPTs, all but two were performed by transplanting the kidney to the left iliac vessels and the pancreas to the right common or external iliac artery through a midline intraperitoneal approach. However, since 7/30/10, nearly all SKPTs were performed with ipsilateral placement of the kidney and pancreas to the right iliac vessels in order to reduce operating time and to preserve the left iliac vessels for future transplantation. All but 5 PTxs were performed from brain-dead donors; 5 SKPTs were performed from donation after cardiac death donors at our hospital in which extracorporeal support was used to assist in management of the donor after declaration of death by cardio-circulatory arrest[11].

Anti-coagulation

Two thousand to three thousand units of intravenous heparin (30-50 units/kg) were administered to SPT and selected SKPT recipients, as a bolus prior to implantation of the pancreas. Following surgery and in the absence of bleeding, patients received a continuous heparin infusion, starting at 300 units/h on day 1, then 400 units/h on day 2, and then 500 units/h on days 3-5 after which time it was terminated[12]. Indications for intravenous heparin included SPT, preemptive SKPT, prolonged pancreas cold ischemia (> 15 h), small or diseased donor or recipient vessels, history of thrombophilia or clotting disorder in the recipient, history of prior pancreas graft thrombosis or extended donor criteria.

Immunosuppression

From 1/02-12/03, 37 patients received depleting antibody induction therapy with 3-5 doses of rabbit antithymocyte globulin (rATG) (1.5 mg/kg per dose); maintenance therapy consisted of tapered steroids, mycophenolate mofetil (MMF) and tacrolimus (TAC)[13]. Subsequently, 16 patients received multi-dose rATG induction, 4 received alemtuzumab (Alem) and rATG, and 5 patients were administered a single dose Alem (30 mg) at the time of transplant. Six of these patients underwent early steroid elimination during this transitional period.

From early 2005 to late 2008, 46 SKPT recipients were part of a prospective trial conducted at WFBH. This undertaking compared a single 30 mg intra-operative dose of Alem to multi-dose rATG (1.5 mg/kg per dose starting intra-operatively) induction. On alternate days, rATG induction was administered (minimum of 3 doses; total cumulative dose 5-6 mg/kg). Both groups received maintenance therapy with early steroid elimination, half-dose MMF (1 gm/d) initially, and full dose TAC (titrated to 12 h trough levels of 8-12 ng/mL)[14].

After completion of rATG, the dose of MMF was doubled to two gm/day. In patients with gastrointestinal intolerance or myelosuppression, the MMF dose was reduced. Corticosteroids were withdrawn after 5 d unless the patient was identified as "high immunological risk", defined by the presence of delayed (kidney) graft function, retransplantation, AA patient < 40 years of age, allosensitization [pre-transplant panel reactive antibody (PRA) level > 20%], or PTA. Since 2009, all patients who receive PTxs at our center (n = 74) have been given single dose Alem induction with MMF, TAC, and either rapid prednisone taper (dose reduction to 5 mg/d by 2 mo following PTx if determined to be high immunological risk), or early steroid elimination[15].

Infection prophylaxis

Fluconazole, valganciclovir, and trimethoprim-sulfamethoxazole were administered to all patients as an anti-infective prophylaxis[7,14]. Cephazolin was used as a peri-operative antibiotic prophylaxis according to the following schedule: (1) A single pre-operative dose; (2) An intra-operative dose; and (3) 2-3 post-operative doses (1 g intravenous).

For at least 12 mo, every Monday, Wednesday and Friday, patients received single-strength trimethoprim-sulfamethoxazole 1 tablet as prophylaxis for Pneumocystis jiroveci. Oral fluconazole (50-200 mg/d) served as an anti-fungal prophylaxis for 1-2 mo. Oral valganciclovir 450 mg/d for 3 mo was the drug of choice as an anti-viral prophylaxis. Dosage was adjusted for either leukopenia or renal dysfunction. If the recipient was at risk for primary cytomegalovirus (CMV) exposure (donor CMV seropositive, recipient CMV seronegative), then oral valganciclovir at a daily dose of 900 mg (with adjustments to dosage as above) was given for a period of 6 mo[7,14].

Peri-operative management

All patients received daily anti-platelet therapy with 81 mg of aspirin. For those patients requiring the post-operative placement of a tunneled central venous catheter, or those requiring prolonged vascular access, a low daily dose of oral warfarin (1 mg) was given to reduce the risk of catheter-associated thrombosis. After insertion of a tunneled subclavian venous catheter, the majority of patients were then sent home on a regimen that included oral electrolyte supplementation and intravenous fluids at home, for a time that was individualized for each patient. Patients were followed closely in the Transplant Outpatient Clinic (at least twice weekly) for the first 3 mo post-transplant and other patient health conditions were treated as indicated.

Diagnosis and treatment of rejection

Elevation in the serum creatinine level of > 0.3 mg/dL without obvious cause triggered the diagnosis of renal allograft rejection, which was made by renal allograft biopsy. The Banff classification was used to determine the severity or grade of rejection[16]. In addition to clinically indicated kidney biopsies, both immediate reperfusion and 1 mo protocol have been performed in SKPT recipients since March, 2008; this, unless there was a specific contraindication. Three steroid boluses and/or oral prednisone recycle were used to treat Banff grade Ia renal rejection episodes. For episodes of acute rejection that did not respond (histologically or clinically) to bolus steroid therapy, rATG rescue therapy was used as the next treatment. Antibody-mediated rejection episodes and Banff grades I b and II grades of rejection were also treated with rATG with the number of doses based on clinical and biochemical parameters. A one month follow-up biopsy was subsequently performed to confirm improved histopathologic changes. The presence of inflammation either on the 1 mo surveillance (subclinical rejection) or follow-up biopsy (persistent rejection) was usually an indication for additional steroid therapy and a subsequent follow-up biopsy.

An unexplained rise in serum amylase, glucose or lipase levels provided clinical suspicion to the diagnosis of rejection of the pancreas graft. Following percutaneous biopsy of the pancreas, the Maryland Classification System[17] was used, initially in the treatment of rejection. More recently, the Banff 2007 schema was utilized[18]. Most grades of pancreas allograft rejection were treated with rATG, while borderline and mild rejection episodes were treated with steroids. In order to document histological improvement and response to therapeutic intervention, follow-up pancreas allograft biopsies were performed. Until there were 2 consecutive biopsies considered as "normal", following SPT, surveillance pancreas biopsies were performed every 3-4 wk[19]. Biochemical parameters were the determinants for clinical biopsies.

Statistical analysis

Both prospective and retrospective databases provided data for compilation. The chi-square test was applied for when variables were categorical, and, with limited data, Fisher's exact test was used. Continuous data were portrayed as means and standard deviations and categorical data were portrayed as percentages and proportions. Significance was ascribed to a two-tailed *P*-value of < 0.05.

RESULTS

From 11/1/01 through 3/1/13, a total of 202 PTxs were performed in 192 patients, including 40 SPTs and 162 SKPTs. The former category included 5 PTA and 35 PAK transplants. 186 PTxs (92%) were primary and 16 pancreas retransplants (10 of which had their primary PTx performed at our center). All but 4 patients received kidney and PTxs either sequentially or simultaneously (one patient received a kidney following a PTA). In addition, 6 patients (3%) underwent subsequent kidney retransplantation. PTx with portal venous and enteric exocrine drainage was performed as intent-to-treat; however, in 23 cases, systemic venous and enteric exocrine drainage was performed (11%) in which portal-enteric drainage was not deemed safe or possible. Indications for systemic-enteric drainage were central obesity (7), difficult vascular anatomy ($n = 7$), and retransplant of the pancreas ($n = 9$), in which the prior PTx was performed with portal venous and enteric exocrine drainage). The incidence of systemic-enteric technique was 7.5% for primary PTxs ($P < 0.0001$) *vs* 56% for pancreas retransplants. The proportion of male recipients (70% *vs* 56%), rate of early relaparotomy (48% *vs* 36%) and recipients $\geqslant$ 80 kg (30% *vs* 24%), were all slightly higher in patients undergoing PTx with systemic venous and enteric exocrine drainage. Rates of early PTx thrombosis were 8% in portal-enteric PTxs *vs* 4% in systemic-enteric (P = NS). Comparable survival rates were found, with an average follow-up of 4.5 years in systemic-enteric *vs* 5.5 years in portal-enteric

PTx recipients, respective patient survival (87% *vs* 86%), PTx graft survival (78% *vs* 62%, $P = 0.165$) and kidney graft survival (78% *vs* 77%).

Pancreas retransplantation

Of the 16 (8%) pancreas retransplants, indications for retransplantation were early thrombosis following SKPT ($n = 9$) or PAK ($n = 1$), primary PTx loss secondary to rejection ($n = 4$), primary nonfunction ($n = 1$), and recurrent auto-immunity ($n = 1$). Types of pancreas retransplants included PTx following SKPT ($n = 10$), second PAK ($n = 3$), second SKPT ($n = 2$), and second PTA ($n = 1$). Eleven patients underwent allograft pancreatectomy prior to retransplantation and 3 at the time of pancreas retransplantation. There were no instances of early PTx thrombosis in pancreas retransplants compared to an incidence of 8.6% in primary PTxs (P = NS). Six patients underwent kidney retransplantation for either early (thrombosis, $n = 1$) or late (chronic allograft nephropathy, $n = 5$, mean 61 mo) graft loss. With a mean follow-up of 72 mo in retransplants *vs* 65 mo in primary PTx, respective patient survival (95% *vs* 86%), PTx graft survival (64% *vs* 65%) and kidney graft survival (82% *vs* 75%) rates were comparable.

Prospective study of alemtuzumab vs rATG induction

In the prospective study of Alem *vs* rATG induction in SKPT, 18 (39%) received rATG induction and 28 patients (61%) received Alem. Enrollment in the two groups was not equal because the randomization schema also included concurrent patients undergoing kidney transplantation alone. Delayed kidney graft function, PRA > 20%, retransplantation, or young AAs (below age 40) were used to identify patients as high immunologic risk, who were managed with chronic steroid therapy ($n = 11$); all other patients were deemed low immunologic risk and underwent early steroid elimination ($n = 35$). Mean follow-up was 5.7 years. With reference to donor, recipient, or transplant characteristics, there were no significant differences between the 2 groups. No differences were noted in one- or five-year patient survival rates. Similarly, one- and five-year uncensored and death-censored kidney and pancreas graft survival rates were comparable. In early PTx thromboses (3.6% Alem *vs* 11% rATG), there were no differences. The same applied to readmissions and other surgical complications between groups. In the Alem group, the overall rates of major infection (39.3% Alem *vs* 66.7% rATG, $P = 0.13$), CMV infection (0 Alem *vs* 16.7% rATG, $P = 0.054$) and acute rejection (21.4% Alem *vs* 44.4% rATG, $P = 0.11$) were slightly lower. In patients with functioning grafts, mean serum creatinine at 1 year (1.1 mg/dL Alem *vs* 1.2 mg/dL rATG) and 5 years (1.4 mg/dL Alem *vs* 1.6 mg/dL rATG), mean calculated aMDRD GFR at 1 year (57 ± 16 mL/min Alem *vs* 55 ± 14 mL/min rATG) and 5 years (55 mL/min Alem *vs* 52 mL/min rATG), glycohemoglobin at 1 year (5.2% Alem *vs* 5.1% rATG) and 5 years (both 5.4%), and mean C-peptide at 5 years (2.2 Alem *vs* 2.3 ng/mL rATG, all P = NS) levels were similar in the Alem and rATG groups.

As a result of this study, we switched from rATG to Alem induction therapy in all of our PTx recipients since 2009.

SKPT in AA recipients

Inferior outcomes following kidney transplantation may be a function of AA ethnicity, but data are limited in PTx. From 11/01 to 3/13, a total of 39 PTxs (1 PTA, 2 PAK and 36 SKPT) were carried out in AA recipients and the other 163 in recipients of other ethnicities (1 Hispanic, 1 Asian, and 161 Caucasian).

Donor and recipient demographics are shown in Table 1. The AA group had a longer duration of pretransplant dialysis (mean AA 32 mo *vs* 16 mo other), fewer preemptive transplants (5.5% AA *vs* 28% other), fewer SPTs (8% AA *vs* 23% other), more patients with a current PRA ⩾ 10% (28% AA *vs* 10% other), more PTxs performed using the systemic-enteric technique (23% AA *vs* 9% other), more patients with 0-1 HLA matches (64% AA *vs* 42% other), and fewer patients who were CMV seronegative (28% AA *vs* 48% other, all $P < 0.05$). Furthermore, the AA group had more patients with a body weight ⩾ 80 kg (51% AA *vs* 24% other), more patients with diabetes for ⩽ 18 years (38% AA *vs* 17% other) and more patients with pretransplant C-peptide levels above 2.0 ng/mL (36% AA *vs* 14% other, all $P < 0.05$).

Outcomes are shown in Table 2. Actual patient (90% AA *vs* 86.5% other), kidney (67% AA *vs* 77% other) and pancreas graft survival (59% AA *vs* 66% other, all P = NS) rates were comparable with a follow-up mean of 67 mo. Early PTx thrombosis rates (10% *vs* 7%) and early relaparotomy (46% *vs* 36%) were likewise comparable in the AA and other groups, respectively. Between groups, cumulative clinical acute rejection rates were similar (33% AA *vs* 27% other).

In AA patients, death-censored dual graft loss was much higher (22% AA *vs* 6% other, $P = 0.01$). In addition, the death-censored kidney graft survival rate (70% AA *vs* 87% other, $P = 0.03$) was lower in the AA group. In AA patients who were pretransplant C-peptide positive ($n = 14$) *vs* C-peptide negative ($n = 25$), there were no differences in mortality (7% *vs* 12%), kidney graft loss (21% *vs* 36%), or pancreas graft loss (36% *vs* 44%) rates, respectively. Based on this analysis, we concluded that PTx in AA recipients was characterized by a higher frequency of detectable HLA antibodies and C-peptide levels at the time of PTx, less HLA-matching, fewer SPTs and PTxs with portal-enteric drainage, and more patients with a type 2 diabetes phenotype. Although rates of survival, acute rejection and pancreas thrombosis were similar, AA patients were at an increased risk for kidney graft loss or dual graft loss compared to other patients in the absence of mortality. This finding may imply either a greater risk for graft loss, better survival in the presence of graft loss, or both, in AA patients.

Table 1 Donor and recipient characteristics in African-American *vs* non-African-American recipients

	AA $n = 39$	Non-AA $n = 163$[1]	*P* value
Donor age (yr)	24.7 ± 10.2	25.2 ± 9.4	NS
Donor BMI (kg/m^2)	23.6 ± 5.4	23.7 ± 2.8	NS
Cold ischemia time (h)	15.8 ± 4.6	16.3 ± 3.8	NS
5-6 HLA-mismatch	25 (64.1%)	68 (41.7%)	0.01
HLA-mismatch	4.8 ± 1.0	4.4 ± 1.2	NS
PRA > 10%	11 (28.2%)	17 (10.4%)	0.008
CMV Recipient negative	11 (28.2%)	78 (47.9%)	0.03
CMV D+/R-	7 (17.9%)	45 (27.6%)	NS
Retransplant	2 (5.1%)	14 (8.6%)	NS
Portal-enteric technique	30 (76.9%)	149 (91.4%)	0.02
SKPT	36 (92.3%)	126 (77.3%)	
SPT	3 (7.7%)	37 (22.7%)	0.04
Recipient age	41.7 ± 9.8	43.0 ± 10.4	NS
Recipient gender: male	20 (51.3%)	94 (57.7%)	NS
Recipient weight ⩾ 80 kg	20 (51.3%)	39 (23.9%)	0.001
Recipient weight	70.9 ± 11.9	71.2 ± 12.7	NS
Dialysis history: SKPT hemodialysis	29/36 (80.6%)	54/126 (42.9%)	
Peritoneal dialysis	5/36 (13.9%)	37/126 (29.4%)	
None (preemptive)	2/36 (5.5%)	35/126 (27.8%)	0.004
Duration of dialysis: SKPT (mo)	31.8 ± 15.1	15.6 ± 17.8	0.02
Duration of pretransplant diabetes ⩽ 18 yr	15 (38.5%)	27 (16.6%)	0.004
Duration of diabetes (yr)	19.7 ± 8.4	26.9 ± 8.6	0.03
Age of onset of diabetes	20 ± 8	16 ± 6	NS
SKPT waiting time (mo)	11.5 ± 6.4	9.7 ± 7.2	NS
C-peptide positive	14 (35.9%)	16 (9.8%)	0.001

[1]161 Caucasian, 1 Asian, 1 Hispanic ethnicity. AA: African-American; BMI: Body mass index; HLA: Human leukocyte antigen; CMV: Cytomegalovirus; PRA: Panel reactive antibody; SKPT: Simultaneous kidney-pancreas transplantation; SPT: Solitary pancreas transplantation; NS: Not significant.

Table 2 Outcomes in African-American *vs* non-African-American recipients

	AA $n = 39$	Non-AA $n = 163$[1]	*P* value
Patient survival	35 (89.7%)	141 (86.5%)	NS
Death with functioning grafts	1 (2.6%)	14 (8.6%)	NS
Kidney graft survival	26 (66.7%)	123/159 (77.4%)	NS
Death-censored kidney graft survival	26/37 (70%)	123/143 (87%)	0.03
Pancreas graft survival	23 (59%)	108 (66.3%)	NS
Death-censored pancreas graft survival	23/37 (62%)	108/148 (73%)	NS
Death-censored dual graft loss	8/37 (21.6%)	9/142 (6.3%)	0.01
Follow-up (mo)	64.9 ± 38.2	69.8 ± 28.6	NS
Relaparotomy	18 (46.2%)	58 (35.6%)	NS
Early thrombosis	4 (10.3%)	12 (7.4%)	NS
Acute rejection	13 (33.3%)	44 (27.0%)	NS

[1]161 Caucasian, 1 Asian, 1 Hispanic ethnicity. AA: African-American; NS: Not significant.

SKPT in "type 2 diabetes"

Over an 11+ year period, we performed 162 SKPTs including 132 in patients with absent or low C-peptide levels (< 2.0 ng/mL, including 21 with measurable C-peptide) and 30 in patients with C-peptide levels ⩾ 2.0 ng/mL (mean C-peptide level 5.7 ng/mL, range 2.1-12.4). At the time of SKPT, patients who were C-peptide positive had a later age of onset of diabetes mellitus (mean age 34 years C-peptide positive *vs* 16 years C-peptide negative, $P = 0.0001$), weighed more (mean 77 C-peptide positive *vs* 69 kg C-peptide negative, $P = 0.27$), had a higher proportion that were age 50 years or older (40% C-peptide positive *vs* 23% C-peptide negative, $P = 0.06$), and had more AAs (47% C-peptide positive *vs* 17% C-peptide negative, $P = 0.001$) compared to those with no or low C-peptide levels. In C-peptide positive patients, diabetes duration was shorter (mean 17 years C-peptide positive *vs* 25 years C-peptide negative, $P = 0.01$) but duration of dialysis was performed over a longer period (median 40 mo C-peptide positive *vs* 14 mo C-peptide negative, $P = 0.14$). The 2 groups did not vary according to dialysis modality or history, sensitization, matching, or

Table 3 Donor and recipient characteristics according to pancreas transplantation category

	SKPT n = 162 in 161 patients[1]	SPT n = 40 in 38 patients[1]	*P* value
Donor age (yr)	27.3 ± 10.6	22 ± 7.6	0.004
Donor BMI (kg/m^2)	23.9 ± 1.4	23.5 ± 6.8	NS
Donation after cardiac death donors	5 (3.1%)	0	NS
Cold ischemia time (h)	16.2 ± 7.4	14.8 ± 3.8	NS
HLA-mismatch	4.5 ± 1.2	2.7 ± 1.5	< 0.001
PRA > 10%	27 (16.7%)	8 (20%)	NS
CMV Donor+/Recipient-	45 (27.8%)	11 (27.5%)	NS
Retransplant	2 (1.2%)	14 (35%)	< 0.001
Portal-enteric technique	147 (90.7%)	32 (80%)	0.09
Recipient age (yr)	42.7 ± 11.3	42.2 ± 8.7	NS
Patients aged 50 or older	42 (26.1%)	8 (21.1%)	NS
Recipient gender: male	94 (58.0%)	19 (50%)	NS
Recipient: AA	36 (22.2%)	3 (7.9%)	0.03
Recipient weight (kg)	71.1 ± 13.5	70.7 ± 12.8	NS
Dialysis history: hemodialysis	82 (50.9%)	NA	
Peritoneal dialysis	42 (26.1%)		
None (preemptive)	37 (23.0%)		
Duration of pretransplant diabetes (yr)	25.3 ± 9.8	26.7 ± 7.7	NS
Waiting time (mo)	10.1 ± 6.3	5.8 ± 7.2	0.002

[1]One patient had 2 SKPTs, two had 2 SPTs, and seven had SKPT followed by SPT. AA: African-American; HLA: Human leukocyte antigen; CMV: Cytomegalovirus; PRA: Panel reactive antibody; SKPT: Simultaneous kidney-pancreas transplantation; SPT: Solitary pancreas transplantation; NS: Not significant; NA: Not available; BMI: Body mass index.

other significant variables.

With a mean follow-up of 5.5 years, patient survival (85% C-peptide negative *vs* 87% C-peptide positive), kidney graft survival (72% C-peptide negative *vs* 77% C-peptide positive), and pancreas graft survival (66% C-peptide negative *vs* 57% C-peptide positive, all P = NS) rates were comparable between groups. Death-censored kidney [both 85% and pancreas (77% C-peptide negative *vs* 61% C-peptide positive, both P = NS)] rates of graft survival were similar between groups. In each group, death-censored dual graft loss occurred in 11%. Rates of early relaparotomy (36% *vs* 33%) and thrombosis (9.8% *vs* 3%) were the same in C-peptide negative and positive groups, respectively. In follow-up, at the five-year point, there were no differences in surgical complications, major infections, HbA1c and C-peptide levels, acute rejection episodes (29% *vs* 30%), readmissions, or renal functional parameters among the 2 groups.

With these findings in mind, C-peptide positive diabetic patients undergoing SKPT appear to have a phenotype consistent with type 2 diabetes (more frequently AA, obese, older, longer duration of pre-transplant dialysis and later age of onset and shorter duration of diabetes) compared to insulin deficient patients at the time of SKPT. However, survival outcomes were comparable. As a result, pretransplant C-peptide levels, provided that they are < 10 ng/mL, are not used solely by us to identify appropriate patients for SKPT.

SKPT vs SPT

We compared outcomes in 162 SKPT and 40 SPT recipients. Demographic characteristics for SKPT *vs* SPT were, in the majority, comparable (Table 3); notwithstanding this, the SPT group had less HLA mismatching (SKPT mean 4.5 ± 1.2 *vs* SPT 2.7 ± 1.5), younger donors (SKPT mean 27 ± 11 years *vs* SPT 22 ± 7.6 years), a lower incidence of AA recipients (SKPT 22% *vs* SPT 8%), shorter waiting time (SKPT mean 10 mo *vs* SPT 6 mo) and an increased number of retransplants (SKPT 1.2% *vs* SPT 35%, all P < 0.05). Outcomes are shown in Table 4. With a mean follow-up of 5.7 years *vs* 7.7 years (P = NS), overall patient (86% SKPT *vs* 87% SPT), kidney (74% SKPT *vs* 80% SPT) and pancreas graft survival (both 65%) rates were comparable.

Mortality was nearly equivalent following either SKPT (13.6%) or SPT (13.2%). No differences in mortality occurred when comparing primary (13.6%) *vs* pancreas retransplants (6.25%, P = NS). However, patterns and timing of death were different as no early mortality occurred in SPT recipients whereas the rates of mortality following SKPT were 4%, 9% and 12%, at 1-, 3- and 5-years follow-up, respectively (P < 0.05). In SPT patients who died, none experienced death with both grafts functioning (DWBGF; 4 had previous kidney graft and 3 previous pancreas graft loss) whereas 15/21 (71%) SKPT recipients experienced DWBGF. In the 26 patients who died, 15 died while both grafts were still functioning, 6 died following pancreas failure, 3 died following kidney graft failure, and 2 died following asynchronous kidney and pancreas graft failure. Secondary to technical issues, 3 SKPT patients died early (within 5 mo) of infection. The remaining 23 deaths occurred at a mean of 53 mo post-transplant (range 6-90). Major causes of late deaths were 7 infectious, 11 cardiovascular, 2 malignancy, and 3 from miscellaneous causes (1 motor vehicle wreck, 1 drug overdose, 1 dialysis withdrawal). Patients aged 50

Table 4 Outcomes according to pancreas transplantation category

	SKPT *n* = 162 in 161 patients[1]	SPT *n* = 40 in 38 patients[1]	*P* value
Patient survival	133/154 (86.4%)	33/38 (86.8%)	NS
Kidney graft survival	120 (74.1%)	28/35 (80%)	NS
Pancreas graft survival	106 (65.4%)	26 (65%)	NS
Follow-up (mo)	68.7 ± 96	92.1 ± 37	NS
Early thrombosis	14 (8.6%)	2 (5%)	NS
Acute rejection	47 (29.0%)	11 (27.5%)	NS
Death in first 4 yr post-transplant	10 (6.2%)	0	NS
Death with functioning grafts	15 (9.3%)	0	0.007

[1]One patient had 2 SKPTs, two had 2 SPTs, and seven had SKPT followed by SPT. SKPT: Simultaneous kidney-pancreas transplantation; SPT: Solitary pancreas transplantation; NS: Not significant.

and older at the time of PTx comprised 42% of those who subsequently died compared to 23% of survivors (*P* = 0.05).

Pancreas graft loss was most commonly associated with death (with a functioning graft), (DWFG) in SKPT recipients whereas acute and chronic rejection accounted for the majority of pancreas graft failures in SPT recipients. Rates of early thrombosis were 8.6% in SKPT and 5% in SPT patients. The overall incidence of clinically evident, pancreas acute or biopsy proven kidney rejection in SKPT was similar to the incidence of clinically evident, biopsy proven pancreas rejection in SPT (SKPT 29% *vs* SPT 27.5%, *P* = NS). As a result of this experience, we concluded that in the setting of careful donor and recipient selection, HLA matching, antibody induction with either rATG or Alem, portal-enteric drainage, flow cytometry crossmatch testing, peri-operative anticoagulation, PTx biopsy monitoring, and TAC/MMF maintenance immunosuppression, similar results can be achieved in SKPT and SPTs.

Experience with allograft pancreatectomy

Of the 202 PTxs, 70 PTx graft losses occurred, of which 21 (30%) resulted in allograft pancreatectomy. Allograft pancreatectomy was performed in 10% of patients; indications were early thrombosis (*n* = 16), late thrombosis (*n* = 2), rejection (*n* = 1), infection (*n* = 1), and pancreatitis/uncontrolled leak (*n* = 1). The incidence of allograft pancreatectomy was 12.5% in pancreas retransplants compared to 10% in primary PTxs. In addition, the incidence was 13% with systemic-enteric drainage compared to 10% with portal-enteric drainage. With a mean follow-up of 70 mo in patients with allograft pancreatectomy compared to 65 mo in PTx recipients without allograft pancreatectomy, respective patient survival (81% *vs* 87%) and kidney graft survival (67% *vs* 76%) rates were comparable. In summary, allograft pancreatectomy was performed in 30% of PTx graft losses, was usually related to early graft loss secondary to thrombosis, and did not appear to impact medium-term patient or kidney graft survival rates.

Outcomes according to different measures of "success"

The definition of PTx graft failure is not uniform and "success" following PTx may be measured by a number of parameters, including freedom from exogenous insulin and dialysis, absence of hyper/hypoglycemia, enhanced well-being and quality of life, and improved life expectancy. With 5.5 years being the mean follow-up, overall patient survival for the entire series (*n* = 192) was 86.5%. A total of 15 patients experienced DWFG whereas 3 patients died following kidney graft failure, 6 following PTx graft failure, and 2 following both kidney and PTx graft failure.

Censored kidney graft survival was 84% and uncensored (actual) was 75%. Reasons for kidney graft failure (*n* = 49) included chronic allograft nephropathy (*n* = 12), DWFG (*n* = 21), polyomavirus nephropathy (*n* = 3), acute/chronic rejection (*n* = 11), and other (*n* = 2). Six patients underwent successful kidney retransplantation, therefore leaving a dialysis-free rate of 87.5% in those patients who survived.

Censored PTx graft survival was 72% and uncensored (actual, insulin-free) was 65%. Reasons for PTx failure (*n* = 70) included acute or chronic rejection (*n* = 30), death with a functioning PTx (*n* = 18), early (*n* = 16) or late (> 3 mo post-PTx, *n* = 3) thrombosis, and infection (*n* = 3). The insulin-free rate among surviving patients was 80%, in view of the fact that a total of 8 patients underwent successful pancreas retransplantation. Among the 30 patients with rejection-based graft failure, 11 were without measureable C-peptide, 4 died, and 15 continued to have measureable C-peptide and had limited pancreas function notwithstanding the fact that all were insulin-requiring. Using the detection of C-peptide for graft survival, the success rate in surviving patients (including pancreas retransplants) was 88% and the death-censored PTx graft survival rate was 80%.

As a result, in patients with severe diabetes, excellent 5 year outcomes following PTx were achieved, as > 86% of patients were still alive, > 87% of survivors were dialysis-free, 88% of survivors had detectable C-peptide levels, and 80% of patients who survived remained insulin-free.

DISCUSSION

The Wake Forest PTx experience in the new millennium is documented herein and chronicles evolving aspects of

recipient selection, technical considerations, immunosuppression, and recipient management protocols based upon numerous prospective and retrospective studies of our own outcomes. Improving outcomes in vascularized PTx are due to a number of factors including reductions in both technical and immunologic graft losses as well as surgical complications. Even with antibody induction and contemporary immunosuppression, when compared to SKPT, SPT is associated with lower pancreas graft survival rates, and higher rates of acute rejection and immunologic pancreas graft loss[1,3-5]. Urinary amylase and serum creatinine levels are unavailable for the diagnosis of rejection in SPTs with enteric exocrine drainage. Moreover, monitoring pancreatic enzymes (lipase and amylase) may not always be reliable. Because of the difficulties in detecting SPT rejection, we advocate protocol pancreas biopsies in these patients[7,19].

Others have reported the value of performing surveillance biopsies of the pancreas allograft as a form of immunologic monitoring[20]. However, in spite of efforts to detect solitary pancreas allograft rejection in a timely fashion, acute rejection episodes occurring late (> 1 year after transplant) are more common in SPT compared to SKPT. Furthermore, the presence of acute rejection and SPT are the two most important risk factors for pancreas graft loss secondary to chronic rejection[21]. We believe that the use of Alem induction coupled with surveillance pancreas biopsy monitoring are reasons why we are able to achieve similar mid-term outcomes in SPT and SKPT[7]. Our data and the experience of others suggests the safety and efficacy of Alem induction in either SKPT or SPT[14,22,23].

A number of recent reports, including our own, have demonstrated the safety and efficacy of SKPT in patients with a type 2 diabetes phenotype[6,7,24,25]. In one series, 94% of recipients of PTxs that were technically successful became completely insulin-free[24]. Long-term results, in type 1 diabetic PTx recipients, were comparable in this study. Ten and twenty year outcomes have been reported by Light *et al*[25,26] from the Washington Hospital Center in either type 1 or type 2 diabetic patients undergoing SKPT. These groups were defined by the presence or absence of C-peptide, respectively. In keeping with our experience, the type 2 diabetic patients were older at the onset of diabetes, had a higher BMI, and contained a higher AA proportion. No differences, similar to our experience, were identified in long-term outcomes in these studies, suggesting that the presence of C-peptide or "type" of diabetes are not important factors in determining recipient selection for SKPT.

We present herein data on 202 PTxs performed at WFBH in the past 11+ years. During this time, we have chronicled a number of changes including: (1) Switching to single dose Alem induction with early withdrawal of corticosteroids in combination with chronic immunotherapy with TAC and MMF dual therapy; (2) Advancing age both in donors and recipients; (3) Transplantation of both the pancreas and kidney on the right side; (4) Immunosuppressive management based on histologic findings with planned implementation of immediate reperfusion kidney biopsies, scheduled pancreas biopsies, as well as clinically indicated and follow-up biopsies; (5) Better understanding of the role of SKPT in patients with a "type 2 diabetes" phenotype; and (6) Reduction in the volume of PTxs in spite of increases in the number of kidney transplants being performed.

Fewer PTxs being performed is not unique to our program but reflects a national trend. There are probably a number of reasons why PTx activity has decreased over time including more restrictive donor selection (and fewer ideal donors), increasing prevalence of obesity among donors and recipients, a number of advances in the medical treatment of diabetes (including new insulin analogues, more sophisticated insulin pumps and glucose sensor devices, better identification and follow-up), financial constraints, and difficulties with access to the waiting list[27,28]. In spite of these drawbacks, whole organ PTx provides an auto-regulating endogenous source of insulin that is able to achieve euglycemia long-term, which in essence renders the patient "ex-diabetic". The goals of PTx include freedom from exogenous insulin, better health and well-being, and improved quality of life and life expectancy. Achieving any of these goals might be a reasonable measure of success.

For patients with end stage diabetic nephropathy, annual mortality on the waiting list over the past decade has ranged from 7% to 10%[29]. Although PTx results in an insulin-free normoglycemic state, these benefits are offset by the potential for surgical complications and the short- and long-term sequelae of chronic immunotherapy, which results in a compression of morbidity. In the future, PTx will remain a useful therapeutic intervention for "complicated" insulin-requiring diabetes because of its metabolic efficiency. Because islet transplant success is defined by C-peptide production and absence of hypoglycemia rather than freedom from insulin therapy and usually involves > 1 donor pancreas, future comparisons of PTx *vs* islet transplant should incorporate similar definitions of graft failure, measures of success, and emphasize longer-term outcomes.

COMMENTS

Background

Vascularized pancreas transplantation (PTx) provides a self-regulating internal source of C-peptide that is consistently able to achieve an insulin-free condition with euglycemia. PTx in diabetic patients is performed in 3 major settings; either before (pancreas transplant alone), after (pancreas after kidney), or concurrent with a kidney transplant (simultaneous kidney-pancreas transplant). The goals of PTx include freedom from exogenous insulin therapy, better health and well-being, and improved quality of life and life expectancy without the need for close glucose monitoring.

Research frontiers

Important areas of research in PTx include targeted or individualized immunosuppression, development of better immune and graft monitoring, improving the donor organ supply, and gaining insights into the pathophysiology of rejection as well as all types of diabetes that result in specific microvascular and metabolic complications.

Innovations and breakthroughs

Success rates for PTx have progressively improved in the past 4 decades, secondary to refinements in diagnostic and therapeutic technologies, improvements in surgical aspects, advancements in therapeutic immunosuppression and anti-infective prevention, new and effective techniques in organ retrieval and preservation technology and increased experience in the selection of donors and recipients. The history of PTx has closely paralleled advances in immunosuppression and surgical techniques.

Applications

In the future, PTx will remain an effective therapy for "complicated" insulin-requiring diabetes because of its metabolic efficiency until new treatments are developed that can achieve normoglycemia without either immunotherapy or major morbidity.

Peer review

Excellent descriptive manuscript of pancreas and kidney transplants.

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RANDOMIZED CONTROLLED TRIAL

Flavonoid-rich beverage effects on lipid profile and blood pressure in diabetic patients

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Abstract

AIM: To compare freeze-dried strawberry (FDS) beverage and strawberry-flavored drink effects on lipid profile and blood pressure in type 2 diabetic (T2D) patients.

METHODS: In a randomized, double-blind, controlled trial, 36 subjects with T2D (23 females; mean ± SE age: 51.57 ± 10 years) were randomly divided into two groups. Participants consumed two cups of either pure FDS beverage (each cup containing 25 g freeze-dried strawberry powder equivalent to one serving of fresh strawberries; intervention group) or an iso-caloric drink with strawberry flavoring (similar to the FDS drink in fiber content and color; placebo group) daily for 6 wk. Anthropometric measurements, 3 d, 24 h dietary recall, and fasting blood samples were collected at baseline and at weeks 6 intervention. After lying down and relaxing for approximately 10 min, each participant's blood pressure was recorded in triplicate with 5 min intervals; recordings were made at baseline and the trial end-point. Each participant's lipid profile was assessed before and after intervention.

RESULTS: Assessment at the weeks 6 intervention showed a significant reduction from baseline in total cholesterol levels and total cholesterol to high-density lipoprotein cholesterol (HDL-C) ratio in the intervention group (179.01 ± 31.86 to 165.9 ± 32.4 mg/L; $P = 0.00$ and 3.9 ± 0.88 to 3.6 ± 0.082 mg/L; $P = 0.00$ respectively), but the change was not significantly different between the two groups ($P = 0.07$, $P = 0.29$ respectively). Systolic blood pressure levels were significantly reduced from baseline in both the FDS and placebo drink groups (129.95 ± 14.9 to 114.3 ± 27.5 mmHg; $P = 0.02$ and 127.6 ± 15.6 to 122.9 ± 14.47 mmHg; $P = 0.00$ respectively), but the reduction was not significantly different between the two groups. Diastolic blood pressure was significantly reduced post-intervention in the FDS drink group compared to placebo group (78.7 ± 7.2 *vs* 84.4 ± 5.8; $P = 0.01$), the reduction was also significant within the FDS drink group (84.2 ± 8.03 to 78.7 ± 7.2; $P = 0.00$). Triglycerides, HDL-C concentrations and anthropometric indices showed no significant differences between or within groups.

CONCLUSION: Short-term FDS supplementation improved selected cardiovascular risk factors in subjects with T2D. Long-term effects on other metabolic biomarkers need to be investigated in future trials.

Key words: Blood pressure; Flavonoid rich beverage;

Lipid profile; Type 2 diabetes

Core tip: Cardiovascular complications are the main cause of mortality in diabetes patients. Considering the role of flavonoids in modulating the latter complications, this study was designed to test the favorable impact of freeze-dried strawberry (FDS) drink, a flavonoid-rich beverage, on the metabolic profile of diabetes patients in a randomized, double-blind, placebo control trial. Lipid profile and blood pressure were improved in patients who consumed the FDS drink for 6 wk. Effects of the latter intervention on other atherosclerotic biomarkers have been discussed separately in *Ann Nutr Metab* 2013; 63: 256-264. This paper describes the further analysis of other metabolic biomarkers.

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INTRODUCTION

The increasing prevalence of type 2 diabetes (T2D) all over the world has highlighted the importance of cost-effective interventions in mitigating the common complications of this devastating disease[1]. Elevated serum triglycerides (TGs), reduced high-density lipoprotein cholesterol (HDL-C), increased blood pressure and enhanced fasting plasma glucose are among the most important complications experienced by patients with diabetes[2]. Diet is known to have a crucial impact on the main risk factors that are responsible for cardiovascular complications in T2D patients, exerting its effects by modulating plasma levels of lipids and lipoproteins, blood pressure, energy balance and oxidative modification or protection of plasma lipids and lipoproteins[3]. Higher consumption of fruits and vegetables are among the dietary recommendations for controlling common complications of T2D[4]. There is scarce evidence for the individual natural components, although flavonoids are thought to play a significant role in health effects of plant-based diets.

The proposed mechanisms underlying the protective role of flavonoids include regulating postprandial glucose, delaying the gastric emptying rate, and reducing active transport of glucose across intestinal brush border membrane. Inhibition of intestine sodium-glucose cotransporter-1 (Na-Glut-1) along with inhibition of α-amylase and α-glycosidase activity makes flavonoids potential candidate factors in the management of hyperglycemia[5,6]. Anthocyanins, a significant group of flavonoids in berries, have been shown to influence glucose absorption, insulin levels/secretion/action, and lipid metabolism, both *in vitro* and *in vivo*[7-9]. Due to high content of essential nutrients and flavonoids, especially anthocyanins, strawberries seem to have relevant biological impacts on human health. Few human investigations have been conducted on the cardiovascular effects of strawberries in T2D patients, despite these patients showing relative risk of cardiovascular disease (CVD) at rates 2- to 4-fold higher than those of non-diabetic subjects[10].

Table 1 Baseline characteristics of the study participants[1]

Characteristic	Intervention $n = 19$	Control $n = 17$	P^2
Age in years	51.9 ± 8.2	51.1 ± 13.8	0.710
Sex, M:F	6:13	5:12	0.433
Weight at study baseline in kg	75.79 ± 9.02	73.38 ± 11.98	0.550
Weight at end-of-trial in kg	75.84 ± 9.04	73.12 ± 11.89	0.750
BMI at study baseline in kg/m^2	27.36 ± 4.23	28.58 ± 4.7	0.330
Duration of diabetes	5.96 ± 5.1	9.00 ± 7.2	0.120
Fasting blood glucose in mg/dL	160.5 ± 51.3	201.7 ± 89.2	0.090
HbA1C, %	7.2 ± 1.6	7.5 ± 1.9	0.740
Waist circumference in cm	99.13 ± 9.06	100.56 ± 8.06	0.680
Hypoglycemic agent use, *n* (%)	17 (89.5)	14 (82.3)	0.423[3]
Anti-hypertension agent use, *n* (%)	5 (26.13)	3 (17.46)	0.253[3]

[1]Values are mean ± SD, unless stated otherwise; [2]Independent *t*-test, unless stated otherwise; [3]χ^2 test.

The main aim in this study was to assess the changes in lipid profile and blood pressure in subjects with T2D after consuming a freeze-dried strawberry (FDS) beverage or placebo drink for 6 wk. A secondary aim of this study was to provide more evidence on the beneficial effects of adding natural flavonoid-rich sources to the diets of diabetic patients and at achievable doses.

MATERIALS AND METHODS

Participants

In order to attribute the effect of FDS beverage more precisely as compared to the flavonoids content of it, a placebo formula was specifically designed with similar fiber and calorie contents. A total of 40 subjects with T2D, aged between 35 and 60 years and with body mass index (BMI) of less than 35 kg/m^2, were selected from Golestan Hospital in Ahavz, Iran for the present investigation. Participants were recruited *via* phone and advertisement. Patients with established T2D (*i.e.*, for over 12 mo) and who had not received any lipid-lowering therapies were recruited to the study. Exclusion criteria consisted of being on medications for any chronic disease (cancer, CVD), smoking (current or stopped for less than 6 mo), lactose intolerance, alcohol consumption of more than 1 oz/d, ingestion of antioxidant supplements and vitamins, being under medical care (including taking medication) for any other disorders. Antidiabetic therapies included metformin, sulfonylurea and glitazone. The basic characteristics of participants are summarized in Table 1.

In order to detect a significance level of $P < 0.05$ and power of 80%, the sample size of 16 was calculated for each group. Considering a dropout rate of 20%, the sample size was increased to 20 for each group. Our intervention was conducted according to the Declaration of Helsinki and all procedures involving human subjects

Table 2 Nutrient composition of freeze-dried strawberry and placebo powders

Nutrient composition of FDS powder	Per 50 g[a]
Carbohydrates in gram	27.1
Protein in gram	4.05
Energy in kcal	108.4
Moisture, %	5
Ash in gram	3.17
Vitamin C in milligram	109.0
Total phenolics in milligram[b]	2006.0
Total anthocyanins in milligram[c]	154.0
Phytosterols in milligram	50
Total dietary fiber in gram	8
Nutrient composition of placebo powder	Per 40 g
Carbohydrates in gram	24
Protein in gram	0
Energy in kcal	98
Total fiber in gram	8
Sugar-free instant drink powder with strawberry flavoring in gram	8

[a]Ten percent fresh weight; Chaucer Foods SA France. Subjects received 50 g/d-approximately 500 g fresh strawberries; [b]Expressed as milligram gallic acid equivalents; [c]Expressed as milligram cyanidin-3-glucoside equivalents. FDS: Freeze-dried strawberries.

were approved by the Medical Research Ethics Committee at Ahvaz JondiShapour University of Medical Science.

Interventional design

This investigation was a double-blind, randomized, controlled clinical trial. A block randomization method was used to randomly assign the matched participants into one of two groups total. Patients were asked to refrain from ingesting flavonoid-rich foods (including other sources of berries, green tea, cocoa and soy products, which were identified for each participant by a screening food frequency questionnaire modified for flavonoids) for 2 wk prior to the study and throughout the intervention period. Subjects were instructed to consume daily either two cups of the FDS beverage (as intervention; containing 25 g pure freeze-dried strawberry powder) or a flavored beverage (as placebo; containing 12 g lactose, 4 g pectin and 4 g sugar-free instant strawberry drink powder) for 6 wk (Table 2). The interval between ingestion of the two cups was at least 6 h and all subjects were also instructed to avoid consuming the strawberry drink with any other snack, lunch or dinner. All participants were asked not to alter their lifestyle throughout the 6 wk trial. The FDS and placebo powders were identical in packaging as well as in taste and color upon dissolving into a glass of water. The researches distributed the FDS and placebo powder packs weekly to the participants. Compliance with the beverage consumption instructions was monitored *via* phone interviews twice a week.

Dietary analysis

Nutrient intake was estimated using a 24 h dietary recall exercise conducted for 3 d at pre- and post-study periods (Table 3). The 3 d averages of energy and macronutrient intakes were analyzed by Nutritionist Pro software (version 3.2, 2007; Axxya Systems, Stafford, TX, United States). All data entry was performed by a trained dietitian. Nutrient information was also obtained through food labels or recipes from participants.

Assessment of variables

Body weight was measured using a scale (Seca, Hamburg, Germany), to 0.1 kg accuracy without shoes. Heights were measured using a stationary stadiometer (Seca), to 0.1 cm accuracy. Systolic and diastolic blood pressures (SBP and DBP respectively) were measured using the Spot Vital Signs device (Welch Allyn, Skaneateles Falls, NY). Participants were asked to lie down and relax for approximately 8 to 10 min, after which three blood pressure measurements were recorded with 5 min intervals.

Clinical analyses

Twelve hour overnight fasting blood samples were collected between 8:00 and 9:00 a.m. Serum and plasma samples were separated by centrifugation at 2000 rpm for 15 min using a 5810R centrifuge (Eppendorf, Hamburg, Germany). The serum samples were stored at -70 ℃ until further assay.

Lipid profiling

Serum concentrations of total cholesterol (TC), TGs, and HDL-C were measured using the standard enzymatic assay kits (Pars Azmoon Co., Tehran, Iran); specifically, TC and TGs were assessed using the cholesterol esterase/cholesterol oxidase method and glycerol phosphate oxidase method, respectively; the HDL-C concentration was measured after precipitation of B-containing lipoproteins.

Supplementary powders, chemicals, and other materials

FDS (intervention) powder was purchased from Chaucer Foods Co. (Paris, France). The flavored beverage (placebo) powder was supplied by Tabriz Chemistry Co. (Tabriz, Iran). All laboratory chemicals were purchased from Farzan Teb Co. (Tabriz, Iran).

Statistical analyses

Data were analyzed using SPSS for Windows (version 16.0; SPSS Inc., Chicago, IL, United States) and the results are expressed as mean ± SE. Normality of the distribution of variables was determined by the Kolmogorov-Smirnov test. The basic characteristics and nutrient intakes of participants in both groups were compared using independent sample *t*-test and χ^2 test. The diabetes medication use in both groups was compared using Mann-Whitney *U* test. Analysis of covariance was used to identify any differences between the two groups post-intervention, adjusting for baseline measurements and covariates. Changes in anthropometric measurements, nutrient intakes and blood lipid parameters of the participants pre- and post-intervention were compared by paired sample *t*-tests. *P* values less than 0.05 were considered as statistically significant.

Table 3 Dietary intake of study participants at baseline and throughout the study[1]

	Run-in period			Throughout the study		
	FDS supplement *n* = 19	Placebo *n* = 17	*P*[2]	FDS supplement *n* = 19	Placebo *n* = 17	*P*[2]
Energy in kcal/d	1760.36 ± 145.21	1697.04 ± 132.42	0.69	1784.03 ± 162.32	1624.42 ± 158.02	0.47
Fat in g/d	75.04 ± 5.17	69.88 ± 7.62	0.96	68.41 ± 4.68	73.21 ± 3.08	0.34
SFA in g/d	22.36 ± 1.65	21.62 ± 1.82	0.72	21.98 ± 1.60	21.23 ± 1.44	0.48
PUFA in g/d	19.39 ± 1.92	16.14 ± 1.51	0.02[3]	19.79 ± 1.74	18.5 ± 1.81	0.46
MUFA in g/d	20.68 ± 1.70	21.32 ± 1.26	0.41	22.56 ± 1.51	21.98 ± 1.42	0.65
Cholesterol in mg/d	173.12 ± 14.23	158 ± 12.16	0.46	169.54 ± 12.50	160.02 ± 14.14	0.94
Dietary fiber in g/d	15.68 ± 1.20	14.73 ± 1.60	0.28	14.25 ± 1.83	14.21 ± 1.40	0.56
Vitamin E in mg/d	3.65 ± 1.72	4.51 ± 1.27	0.35	4.79 ± 1.50	4.15 ± 1.42	0.65
Vitamin C in mg/d	71.25 ± 25.02	68.42 ± 18.12	0.75	64.54 ± 16.32	69.47 ± 21.56	0.48
Zinc in mg/d	8.24 ± 1.32	9.80 ± 1.42	0.43	7.53 ± 1.25	8.67 ± 1.36	0.09

[1]Data are mean ± SD; [2]Obtained from independent sample *t*-test; [3]Significant difference between groups; SFA: Saturated fatty acid; PUFA: Polyunsaturated fatty acid; MUFA: Monounsaturated fatty acid; FDS: Freeze-dried strawberry.

Ethics approval

The study protocol was approved by the Medical Ethics Committee of Ahvaz JondiShapour University of Medical Sciences (Study No. ETH_393). The clinical trial registration number is IRCT201110117765N1.

RESULTS

All participants completed the study, but 4 people were excluded from the statistical analysis. Among those 4 excluded patients, 3 from the placebo group experienced changes in medication or became uninterested in the taste of beverage and 1 did not consume the FDS drink due to unwillingness to continue (Figure 1). Except for the temporary gastrointestinal discomfort reported by some patients in both groups, all cases of which were alleviated during the first week, the participants demonstrated good compliance with the FDS and placebo beverage consumption.

Table 1 presents the baseline characteristics of the participants in the study groups. The two groups were statistically similar in most baseline characteristics. Weight and BMI remained unchanged during the study for both groups. No statistically significant difference was seen within and between groups in micro- and macro-nutrients dietary intake, except for polyunsaturated fatty acids intake at the beginning of intervention and at the end of the study, for which the difference in terms of dietary intake remained insignificant (Table 3).

Lipid profile

The lipid profiles were not significantly different between the FDS and placebo groups at baseline. Results of covariance analysis showed statistically significant differences within the FDS group for TC ($P = 0.000$) and TC:HDL-C ratio ($P = 0.002$) at the end of study, adjusted for monounsaturated fatty acid intake (Table 4). FDS beverage consumption caused a 13.8% decrease in TC and a 7.1% decrease in TC:HDL-C ratio compared to baseline (Figure 2). No significant differences in the lipid profiles were observed between the two groups at baseline and 6 wk post-intervention (Table 4).

Blood pressure

SBP was significantly decreased in both the FDS and placebo groups, compared to baseline. DBP was also significantly reduced in the FDS group compared to the placebo group (Table 4).

DISCUSSION

The potential role of berries, a natural source of flavonoids, in improving lipid profile has been indicated by an emerging body of evidence. Strawberry puree supplementation in combination with other berries has been shown to increase HDL-C and decrease SBP (*vs* a control group) in subjects with cardiovascular risk factors[11]. Yet, scant human interventions have been carried out in order to prove this protective role of berries in subjects with diabetes. In order to confirm the recommendation of adding two servings of fruits with low glycemic index for proper control of diabetic complications[12], we tested a 50 g freeze-dried strawberry powder (equivalent to approximately 500 g or two servings of fresh strawberries) to investigate the beneficial effects of strawberries in a standard freeze-dried form on lipid profile and blood pressure levels in subjects with T2D. The effects of FDS beverage consumption on glaciated hemoglobin and atherosclerosis biomarkers in this study have been indicated in a separate paper[13].

In previous studies[11,14,15], plain water was mainly used as the placebo beverage; however, for better elucidation of the role of polyphenols content of berries, we used a fiber- and energy-matched placebo powder. To our best knowledge, this is the first double-blind, placebo controlled trial carried out with iso-caloric/fiber placebo beverage, investigating favorable effects of FDS beverage in T2D patients. Results from previous *in vitro* studies indicate that anthocyanin might affect expression of genes involved in cell cycling, signal transduction, and lipid and carbohydrate metabolism in adipose tissue cells[8,9,16].

Clinical trials involving cranberry and mixed ber-

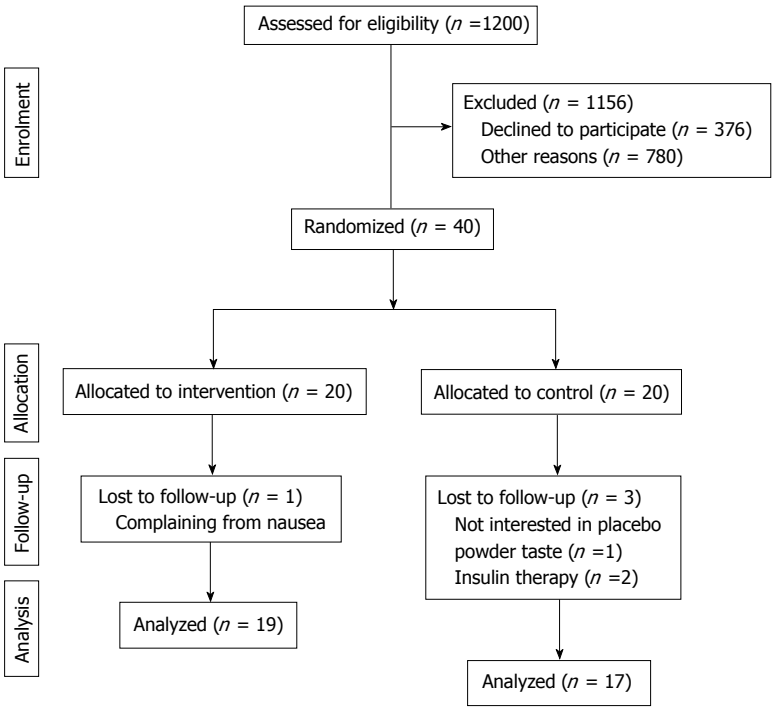
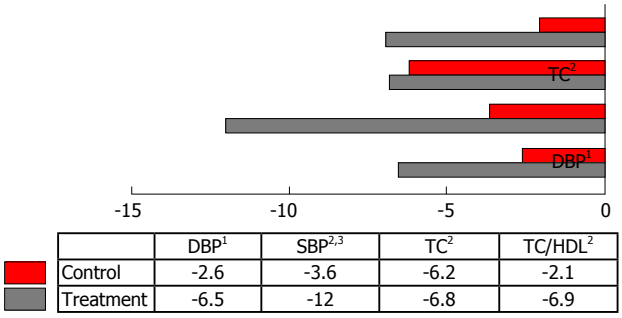


Figure 1 Summary of patient enrollment.



	DBP[1]	SBP[2,3]	TC[2]	TC/HDL[2]
Control	-2.6	-3.6	-6.2	-2.1
Treatment	-6.5	-12	-6.8	-6.9

Figure 2 Percentage of change in total cholesterol, total cholesterol/high-density lipoprotein-cholesterol, systolic and diastolic blood pressures after 6 wk post-intervention in both the freeze-dried strawberry and placebo group. [1]Significant reduction in the FDS group compared to the placebo group; $P = 0.003$ *vs* $P = 0.134$; [2]Significant reduction within the FDS group in TC, TC/HDL and DBP; $P = 0.000$, $P = 0.002$ and $P = 0.023$ respectively; [3]Significant reduction within the placebo group, $P = 0.007$. TC: Total cholesterol; TC/HDL-C: Total cholesterol/high-density lipoprotein-cholesterol; DBP: Diastolic blood pressures; SBP: Systolic blood pressures; FDS: Freeze-dried strawberry.

ries extract supplementations have led to improved dyslipidemia in T2D patients and patients with hyperlipidemia[17,18]. The 6 wk FDS supplementation also improved glycated hemoglobin (HbA1c) in our intervention study[13]. However, in the present study, no significant changes were observed in low-density lipoprotein-cholesterol (LDL-C) and HDL-C after the 6 wk supplementation with FDS or placebo beverage. These findings might be due to near-normal baseline levels of LDL-C and HDL-C in our intervention and control groups. Decreases in plasma TC and the TC:HDL-C ratio were significantly greater in the FDS-supplemented group compared to the baseline (Figure 2). Our findings are similar to the previous studies reporting the effects of freeze-dried strawberries in lowering TC and LDL-C in subjects with metabolic syndrome[11,14,15].

The change in lipid profile was not significant between the intervention and control groups in this study, which might be due to the similar fiber content of the placebo drink and the FDS beverage. However, this study was specifically designed to assess the effects of the flavonoids content of the FDS beverage. Further investigations with a fiber-free placebo (as a third group) are needed to study the favorable effects of the whole content of berry products in diabetic patients.

The FDS supplementation in this study significantly decreased SBP and DBP (Table 4). These findings are in agreement with the results from a study, in which the anti-hypertensive effects of freeze-dried blueberries were assessed in obese subjects with metabolic syndrome or of mixed berry supplementation in those subjects with CVD risk factors[17,19,20]. Although, some studies have shown no significant changes in blood pressure after FDS supplementation in subjects with metabolic syndrome, which might be due to smaller sample size and/or shorter duration of intervention[14,15].

The impact of berries or anthocyanin in mitigating hypertension has been explained as enhancing endothelial nitric oxide synthase levels in endothelial cells, decreasing vasoconstriction *via* nitric oxide-mediated pathway, and reducing renal oxidative stress[16,17,21,22]. SBP was also significantly decreased in the control group at 6 wk post-intervention (Table 4). The latter might be attributable to the effects of the soluble fiber content of the placebo drink, indicating the possible role of fiber in FDS beverage, which could partially contribute to the

Table 4 Metabolic variables at baseline and 6 wk after flavonoid-rich or placebo supplementation in both groups

	Groups		
	Intervention *n* = 19	Control *n* = 17	*P*[a]
TC in mg/L			
Baseline	179.01 ± 31.86	196.35 ± 50.5	0.19
6 wk	165.9 ± 32.4	183.29 ± 49.9	0.07
Change 0-6 wk	-13.1 ± 16.45	-13.05 ± 42	0.80
%CI for change	-7.57 to 20.32	-8.5 to 34.67	
P for change within group	0.000[1]	0.216	
LDL-C in mg/dL			
Baseline	95.84 ± 26.45	116.51 ± 48.8	0.13
6 wk	92.96 ± 28.03	108.19 ± 40.2	0.19
Change 0-6 wk	-2.87 ± 0.47	-8.3 ± 0.13	0.60
%CI for change	-6.8 to 12.53	-15.52 to -32.17	
P for change within group	0.54	0.46	
HDL-C in mg/dL			
Baseline	47.38 ± 13.67	46.54 ± 12.32	0.84
6 wk	48.36 ± 12.62	47.7 ± 12.26	0.88
Change 0-6 wk	0.97 ± 2.4	1.2 ± 3.1	0.78
%CI for change	-2.1 to 0.18	-2.8 to 0.38	
P for change within group	0.098	0.12	
TGs in mg/dL			
Baseline	184.6 ± 87.6	195.2 ± 84.2	0.81
6 wk	166.37 ± 99.59	183.2 ± 84.4	0.65
Change 0-6 wk	-18.28 ± 58.7	-11.88 ± 90.56	0.80
%CI for change	-10.5 to 46.6	-34.6 to 58.4	
P for change within group	0.19	0.59	
TC/HDL-C			
Baseline	3.9 ± 0.88	4.4 ± 1.5	0.19
6 wk	3.6 ± 0.82	4.3 ± 1.2	0.29
Change 0-6 wk	-0.28 ± 0.35	-0.35 ± 0.08	0.40
%CI for change	0.11 to 0.45	-0.06 to 1.01	
P for change within group	0.002[1]	0.08	
LDL-C/HDL-C			
Baseline	2.1 ± 0.68	2.6 ± 1.2	0.16
6 wk	1.9 ± 0.62	2.3 ± 0.94	0.24
Change 0-6 wk	-0.12 ± 0.36	-0.27 ± 0.06	0.57
%CI for change	0.11 to 0.45	-0.06 to 1.01	
P for change within group	0.183	0.33	
SBP in mmHg			
Baseline	129.95 ± 14.9	127.6 ± 15.6	0.74
6 wk	114.3 ± 27.5	122.9 ± 14.47	0.25
Change 0-6 wk	-15.94 ± 27.98	-4.7 ± 6.2	0.57
%CI for change	2.45 to 29.43	1.49 to 7.91	
P for change within group	0.023[1]	0.007[1]	
DBP in mmHg			
Baseline	84.2 ± 8.03	86.76 ± 6.3	0.168
6 wk	78.7 ± 7.2	84.4 ± 5.8	0.014[1]
Change 0-6 wk	-5.5 ± 7	-2.3 ± 6.7	0.16
%CI for change	0.11 to 0.45	-0.06 to 1.01	
P for change within group	0.003[1]	0.134	

[1]*P* value is regarded as significant; [a]*P* value between groups, *P* value < 0.05 is regarded as significant. Values are mean ± SD. TC: Total cholesterol; LDL-C: Low-density lipoprotein-cholesterol; HDL-C: High-density lipoprotein-cholesterol; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglyceride.

reduction in SBP.

It should be mentioned that the lack of a dose-response treatment in a cross-over intervention and of the use of more sensitive biomarkers are among our study's limitations. Gastrointestinal discomforts were anticipated, considering the excessive fiber intake accompanying the placebo drink[6]. Those who completed the entire 6 wk study period experienced this temporary gastrointestinal discomfort during the first week, which was alleviated thereafter (but which equated to a 15% drop-out rate). However, the FDS beverage was well tolerated by participants (with only a total 5% drop-out) rate. It is likely that the administration of the FDS or placebo beverage in two equal doses throughout the day and the instruction of participants to avoid consuming the drinks along with a main meal or other snacks contributed to the good tolerance. Precise adjustment for total fiber intake, longer duration of intervention, and administration of freeze-dried berry products in three or four doses throughout the day could improve tolerability while exerting more beneficial effects in future investigations.

In conclusion, our study suggests a cardio-protective role of dietary achievable doses of strawberries in subjects with T2D. These findings justify further research to provide more evidence to support the inclusion of strawberries as a part of healthy dietary practices for diabetic patients.

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COMMENTS

Background

Increasing prevalence of type 2 diabetes (T2D) has lead to a great focus on reasonable interventions for mitigating its disease-related complications. Diet has a crucial impact on the main risk factors of cardiovascular complications in T2D patients. Flavonoids, as a natural component of a plant-based diet, might play a significant role in improving the complications of T2D. Still there is a need for more precise controlled trials on cardiovascular effects of these sources, such as berries, in diabetic patients.

Research frontiers

Strawberries, as a rich source of flavonoids, may have biological impacts on human health through their inhibition of the main mechanism in hyperglycemia and improving blood pressure. This study was aimed to provide more evidence to support the beneficial effects of adding natural flavonoid-rich food sources at dietary achievable doses in diabetic patients. The authors investigated the changes in lipid profile and blood pressure after consumption of a freeze-dried strawberry (FDS) beverage or placebo drink by diabetic patients.

Innovations and breakthroughs

Beneficial effects of flavonoids on cardiovascular complications have emerged as a subject of considerable research interest. This study, therefore, was carried out to investigate effects of FDS beverage on lipid profile and blood pressure in comparison to a placebo drink that was specifically designed to resemble the FDS beverage in taste, color, and fiber and energy content, after a 6-wk course of supplementation in patients with diabetes. This is the first time that a randomized controlled trial has been carried out on the effect of FDS on T2D complications.

Applications

Considering the favorable effects observed upon adding two servings of fruits with low glycemic index to the dietary plan of diabetic patients, this study might suggest a suitable method of supplementing the daily dietary plan of such patients with flavonoid-rich fruits and beverages.

Terminology

FDS is a term used to describe organic strawberries that have been dried using the freeze-drying technique, which is considered the most effective method for protecting the micronutrients and phytochemical content of fruits and veg-

etables under drying conditions. Freeze-drying enables us to take advantage of using flavonoid-rich fruits and vegetables while sustaining the highest possible quality during every season.

Peer review

This study is the first randomized control trial that has been carried out to study the effects of FDS on T2D mellitus complications. Lipid profile and blood pressure were improved in patients who consumed the FDS beverage for 6 wk. The study is interesting because it demonstrates the efficacy of dietetic changes related to atherosclerosis in patients affected with T2D mellitus.

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 200201 03498. 2002 Aug 1

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H. pylori*, *E coli*, *etc.*

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