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THERAPEUTIC AND DIAGNOSTIC GUIDELINES

Feasibility of large experimental animal models in testing novel therapeutic strategies for diabetes

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Abstract

Diabetes is among the top 10 causes of death in adults and caused approximately four million deaths worldwide in 2017. The incidence and prevalence of diabetes is predicted to increase. To alleviate this potentially severe situation, safer and more effective therapeutics are urgently required. Mice have long been the mainstay as preclinical models for basic research on diabetes, although they are not ideally suited for translating basic knowledge into clinical applications. To

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validate and optimize novel therapeutics for safe application in humans, an appropriate large animal model is needed. Large animals, especially pigs, are well suited for biomedical research and share many similarities with humans, including body size, anatomical features, physiology, and pathophysiology. Moreover, pigs already play an important role in translational studies, including clinical trials for xenotransplantation. Progress in genetic engineering over the past few decades has facilitated the development of transgenic animals, including porcine models of diabetes. This article discusses features that attest to the attractiveness of genetically modified porcine models of diabetes for testing novel treatment strategies using recent technical advances.

Key Words: Pancreatic islet; Diabetes mellitus; Pig; Transgenic; Genetic engineering; Transplantation; Xenotransplantation

Core Tip: Safer and more effective therapeutics are urgently required for managing the diabetes epidemic. Mice have been used predominantly as preclinical models for basic research on diabetes, although murine models are not ideally suited for translating basic knowledge into clinical applications. This article discusses features that attest to the attractiveness of genetically modified porcine models of diabetes for testing novel treatment strategies using recent technical advances.

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INTRODUCTION

Diabetes is a profligate disease that is characterized by disordered glucose metabolism that results from absolute or relative deficiency of insulin. Diabetes is among the top 10 causes of death in adults and was estimated to have caused four million deaths globally in 2017[1]. Globally, there are approximately 500 million individuals with diabetes, and this number is expected to increase by 25% and 51% by 2030 and 2045, respectively. Worldwide, diabetes imposes a large economic burden on healthcare systems, and the estimated annual global health expenditure attributable to the disease ranges from USD 612 to 1099 billion. Thus, diabetes has a major impact on the lives and well-being of individuals, families, and societies. The prevention and effective management of diabetes should be considered a public health priority to reduce the financial burden[2].

TYPE 1 DIABETES

The three main types of diabetes are type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and gestational diabetes mellitus. T1DM is characterized as a multifactorial autoimmune disease that occurs due to a specific immune-mediated permanent destruction of pancreatic β-cells and results in a lifelong dependence on exogenous insulin. Approximately 5%-10% of patients with diabetes have T1DM, and the incidence and prevalence of T1DM is predicted to increase[3-5]. Worldwide, approximately 78000 youth are diagnosed with T1DM annually[6,7]. T1DM increases the risk of unstable glycemic control, hypoglycemia unawareness, and even sudden unexplained death[8], and induces pathological alterations including microvascular and macrovascular complications. The Diabetes Control and Complications Trial (DCCT) Research Group showed that the severity and duration of hyperglycemia exposure are

directly related to the risk of development and progression of microvascular complications such as retinopathy and diabetic kidney disease in T1DM, both in adolescents and adults[9]. Individuals thus affected with end-stage kidney disease often require hemodialysis and, eventually, kidney transplantation to manage renal failure[10]. Macrovascular complications, cerebrovascular disease, and peripheral vascular disease resulting from atherosclerosis are the leading causes of morbidity and mortality in adults with T1DM[11,12]. Thus, T1DM is a serious disease that confers not only an economic burden but also psychological distress for patients over a long period.

Treatment of T1DM

Exogenous insulin therapy: Most T1DM patients require intensive insulin regimens, *via* multiple daily injections of prandial and basal insulin or continuous subcutaneous insulin infusion (CSII), to maintain euglycemia[13,14]. The DCCT Research Group demonstrated that intensive insulin regimens achieved near-normal glycemic control and reduced the risk of the onset and progression of T1DM-related complications[15]. Some trials of novel technological T1DM treatments have been launched and have yielded good results. The combined use of continuous glucose monitoring devices with insulin pumps has enabled the development of automated insulin delivery systems known as closed-loop devices, with predicted low-glucose suspension that has reduced the incidence of hypoglycemia in children and adolescents in clinical studies[16-19]. Furthermore, relative to conventional and sensor-augmented insulin pump therapy, the bi-hormonal bionic pancreas was able to achieve superior glycemic regulation without the need for carbohydrate counting[20,21]. The technology was established using a large animal diabetic porcine model[22,23] and was subsequently applied in a clinical trial[21]. Automated glycemic control is progressive, but it carries some risks. With closed-loop devices, T1DM patients and their families need to expend painstaking efforts to count carbohydrates, closely monitor blood glucose, and make dosing decisions for insulin—a drug with a narrow therapeutic range and a low margin for error. Hyperglycemia with ketosis due to pump infusion-set failure is another possible issue[17]. Thus, automated insulin delivery requires patient and provider education to optimize its outcomes[6,7,16,21,24]. Another important current limitation is that subcutaneously administered insulin, either by an CSII device (as part of a closed-loop system) or through multiple injections, will incur a lag time to initiate pharmacological action[25-27]. In studies of bi-hormonal closed-loop devices, the results represent advances in care, but remain cumbersome, imprecise, and costly. Indeed, loss of wireless connectivity occurs up to 4% of the time[21]. To date, no protocol has been established to eradicate exogenous insulin therapy entirely without substantial recipient risk. Thus, research needs to be continued to produce more suitable physiological therapies. An effective alternative approach is β-cell replacement therapy.

β-cell replacement therapy: Human whole pancreas or islet transplantation may constitute a life-saving therapy for T1DM patients. These therapies provide considerable advantages for diminishing the total daily insulin dose and lowering the frequency of hypoglycemic reactions[28,29]. Pancreas transplantation has been accepted as a proven therapy, and simultaneous pancreas-kidney transplants function for an average duration of 9 years[30]. However, organ transplantation is an insufficient treatment despite the fact that patients undergo major surgery, need lifelong immunosuppressive therapy and hold incidental risks of infection, cancer, and nephrotoxicity constitute additional challenges. Islet transplantation was performed on an experimental basis since its introduction by Shapiro *et al*[31] in 2000 as a feasible clinical procedure. Islet transplantation has an advantage in that it does not require major surgery. Moreover, the treatment methods have been refined substantially, including more optimal islet preparation, culture, safer transplant techniques, and more effective anti-inflammatory and immunomodulatory interventions, over the past decade[32]. Islet transplantation therapy can be maintained for 3 years, an average, without insulin in specialized protocols[33]. A multicenter, single-arm, phase 3 study of an investigational product containing purified human pancreatic islets was conducted at eight centers in North America[34]. The success reported with this study has established a proof of principle for cellular transplantation, although the treatment still has several hurdles to cover to reach clinical application in real-world clinical practice (Table 1).

Table 1 Hurdles in clinical islet transplantation for type 1 diabetes mellitus

	Hurdles in clinical islet transplantation for type 1 diabetes mellitus
1	Poor access to human islets due to the scarcity of organ donors
2	Graft failure (*e.g.*, metabolic pressure, oxidative stress caused by hypoxia or inflammation)
3	Continued autoimmunity and alloimmunity
4	Immunosuppressive drug therapy
5	Behavior of the transplanted cells in the growing body for transplantations performed in adolescents/children

EXPERIMENTAL ANIMAL MODELS FOR TESTIG NOVEL TREATMENT STRATEGIES

The development of therapeutic strategies for T1DM is progressing, although all of them have some imperfections. Thus, safe and potent therapeutics need to be developed to combat diabetes. Animal models are indispensable for the discovery, validation, and optimization of novel therapeutics for safe human use. Mice have long been the mainstay of preclinical research and are a widely used mammalian species in biomedical research, mainly because they are convenient and cheap to house and as methods for their genetic modification are well advanced[35,36]. However, the translational value of murine models is limited due to their distinct anatomy and physiology[37]. The murine models cannot replicate the complications of organ and/or cell transplantation, such as blood vessel and bile duct injuries, that might occur in human allotransplantation in T1DM patients. Experimental procedures and conditions should be accurately described to improve their reproducibility and to facilitate the translation of findings in preclinical animal models.

ATTRACTIVENESS OF LARGE EXPERIMENTAL AINMAL MODEL IN MEDICAL RESEARCH

It is preferable that such investigations are conducted on animal species that have anatomical and physiological similarities to humans. Large animals, especially pigs, are well suited for developing and refining biomedical procedures and medical equipment for biomedical research as they share many similarities with humans, including body size, anatomical features, physiology, and pathophysiology. Moreover, it is well established that the pharmacokinetics of orally or subcutaneously administered compounds in pigs are similar to those of humans[38]. In addition, pigs are monogastric omnivores and are likely to be obese as well as dyslipoproteinemic, similar to humans. Other characteristics of pigs include the fact that they mature relatively quickly for a large species (6-7 mo), have a short gestation period (approximately 114 d) and high fecundity (8-14 offspring per litter), and their long-life cycle and high litter size make the production of genetically modified pigs less time-consuming compared to other animal species[38,39]. Furthermore, there is wide public acceptance of the humane use of pigs in research, unlike that for other nonrodent species, such as primates. Genetically modified animals are vital for gaining a proper understanding of disease mechanisms and for developing novel therapies. The extension of genetic modification technology to pigs has greatly increased their value in biomedicine, motivating efforts to develop porcine models that replicate human diseases, including cardiovascular and neurodegenerative diseases, neoplasms, and diabetes[35]. Pigs are now considered promising models to overcome gaps between proof-of-concept models, to bridge the gap between bench and bedside, and as precursors to clinical studies[21-23]. The major advantages and disadvantages of pigs in medical research are listed in Table 2.

MODELING DIABETES IN PIGS

Porcine insulin differs by only one amino acid from human insulin and was widely used to treat T1DM before human insulin could be produced in large quantities by recombinant DNA technology[40,41]. Pigs have similar pancreatic and islet structure,

Table 2 Use of pigs as biomedical research subjects

	Advantages
1	Similar body size, shape, and anatomy as that of humans
2	Human-relevant metabolic physiology and pathophysiology
3	Monogastric omnivore
4	Multiparity, short gestation, short generation interval, and long-life cycle
5	Pancreatic and islet architecture similar to that of humans
6	Can undergo the same surgical procedure as in humans
7	Tools for genetic alterations are available
8	High litter size makes the production of genetically modified pigs less time-consuming in comparison with other livestock species
	Disadvantages
1	Specialized facilities are required
2	Costly to maintain
3	Ethical issues associated with the use of pigs in biomedical research

total β-cell mass, ratio of β-cell mass to body mass, and β-cell replication capacity to that of humans[42,43]. Thus, pigs serve as tissue and organ donors for β-cell replacement therapies in T1DM[44-46]. Pigs are suitable for testing medical devices and surgical techniques, such as pancreatectomy and transplantation of the pancreas and islets, to develop and refine treatment methods for T1DM.

RESERCH ETHICS IN PIG MODELS

Species selection for research must be based on ethical standards and conducted on a case-by-case basis such that the benefits are assessed according to the predictability of the animal model for the specific function. In the United States, the 1966 Animal Welfare Act is the federal law that regulates how animals must be treated in research; however, it does not apply to animals raised for food[47,48]. The European Union's (EU) Directive on the Protection of Animals Used for Scientific Purposes largely aims on eliminating or reducing the potential pain and distress of live animals included in research and also excludes the animals raised for food[48]. The response from ethics or regulation would most certainly have been the same between the United States and Europe. We reject the general argument that pigs are completely suitable for our experiments. The decisions about choice of species are complex and has evolved with new knowledge. In a scientific sense, as mentioned previously in this article, the use of pigs for this study is based on their many similarities to humans. On the contrary, in an ethical context, the potential scientific, technical, and economic benefits, all set against the need to minimize harm. The most effective application of the principles of practical ethics to the conduct of experiments with animals, which we used in our study, is the concept of the three R's (reduction, replacement, and refinement) introduced by Russell and Burch[49,50]. The regulated procedures state that the research must include minimum number of animals; involve animals with the lowest degree of neuro-physiological sensitivity; cause the least pain, suffering, distress, or lasting harm; and are most likely to produce satisfactory results. Legislation on the protection of vertebrate animals used for experimental and other scientific purposes has been formulated in line with the three R's. It was first presented in European Union Directive 86/609/ECC (EU Directive European Union Directive 86/609, 1986)[51-54]. Regardless of the species involved, proper care and management are essential for the well-being of the animals, validity of the research data, and health and safety of animal care personnel. Therefore, all our animal experiments in this study were carefully inspected and approved by Meiji University's Institutional Animal Care and Use Committee (IACUC). All animals were housed and maintained in accordance with the IACUC guidelines. All animal care and experimental procedures were performed in accordance with the regulations contained in the Japanese Act on Welfare and Management of Animals. The pigs were housed in a temperature-controlled room, with free access to water and growth-stage appropriate commercial feed and observed

by an animal husbandry personnel under the supervision of an attending veterinarian on a daily basis.

METHODS TO INDUCE DIABETES IN PIG

Surgery

Diabetes can be surgically induced in pigs by pancreatectomy to ablate endogenous insulin production. The benefit of this method is the lack of toxic adverse effects on other organs. However, the prerequisites of highly specialized training, surgical equipment, confounding effects of eradicating exocrine pancreatic digestive enzymes, and other islet hormones are some of the disadvantages with this method.

Chemical induction

Chemical approaches for diabetes induction, such as use of the diabetogenic drugs streptozotocin or alloxan, target insulin-secreting β-cells. Both drugs are cytotoxic glucose analogs that have a high affinity for the glucose transporter 22 in β-cells. Diabetes can be induced by daily injection of the β-cell cytotoxin streptozotocin (0.1 mol/L at a dose of 50 mg/kg) for 3 d, resulting in a > 80% β-cell reduction, an increase in the plasma glucose to diabetic levels, and hypertriglyceridemia. Chemical induction is the standard model of diabetes in rodents, but it is often difficult to use in large animals due to reduced efficacy, relevant side effects, and an unacceptable mortality rate. Multiple injections and higher drug doses are needed for the induction of diabetes in larger animals; thus, chemical induction is an unstable method for the induction of diabetes in pigs[38,55].

Genetic engineering

Genetic engineering of pigs is a remarkably refined approach for generating tailored porcine diabetes research models. Transgenic (Tg) pigs are an attractive model for the analysis of pancreatic development and for testing novel diabetes treatments. Several groups have generated porcine diabetic models. Renner *et al*[56] generated pigs with T2DM that could express a human dominant-negative incretin hormone–glucose-dependent insulinotropic polypeptide receptor mutant (GIPR^{dn}) in pancreatic islets controlled by the rat insulin promoter. A permanent neonatal diabetes pig model was established by generating Tg pigs that expressed a mutant porcine *INS* (insulin) gene (INS C94Y) orthologous to human INS C96Y[57-60]. However, no diabetes-associated renal pathological changes were detected in these models. Diabetes has a multitude of phenotypic manifestations that are unlikely to be recapitulated in a single animal model. Pancreatic duodenum homeobox 1 (*PDX1*) expression is crucial for pancreatic organogenesis and is a key regulator of insulin gene expression[61]. Using genome-editing technologies, *PDX1*-knockout (KO) pigs are generated; however, *PDX1*-KO is fatal[62-64]. Nonetheless, a few of *PDX1*-modified pigs generated by the clustered regularly interspaced short palindromic repeat/clustered regularly interspaced short palindromic repeat/CRISPR-associated proteins9 (CRISPR/Cas9) system that is introduced into zygotes can survive[65].

Diabetic Tg pigs generated in our laboratory

Tg-cloned pigs with a mutant human hepatocyte nuclear factor 1α gene: Maturity-onset diabetes of the young (MODY3) is characterized by impaired insulin secretion, with less impact on insulin action, and is commonly caused by dominant-negative mutations in the gene encoding hepatocyte nuclear factor 1α (*HNF-1α*)[66,67]. MODY3 is a noninsulin-dependent type of diabetes with autosomal dominant inheritance wherein *HNF1α* gene mutations lead to pancreatic β-cell dysfunction and impaired insulin secretion. We generated a pig model for MODY3 by expressing a mutant human *HNF1*α gene (HNF1α P291fsinsC) using intracytoplasmic sperm injection (ICSI)-mediated gene transfer (MGT) and somatic cell nuclear transfer (SCNT)[68-70]. After we generated the Tg pigs, their sperm was frozen and frozen sperm heads that were thawed subsequently and preincubated with the gene construct were microinjected into oocytes for fertilization, resulting in the incorporation of foreign genes into the genome of the host egg. A system for the mass *in vitro* production of mature eggs with high developmental ability in pigs has been established. Therefore, we could create Tg pigs as needed. Piglets developed hyperglycemia at 2 wk, and showed glomerular nodular lesions in the kidneys, a hallmark of diabetic nephropathy (described in the section of Microvascular and Macrovascular Complications) at 19 wk

that further expanded over the 10-mo observation period[69,70]. Furthermore, Tg pigs manifest diabetic retinopathy and cataracts, similar to those in T1DM patients[70].

Tg cloned pigs carrying *PDX1*-Hairy and enhancer of split 1: Hairy and enhancer of split 1 (HES1) control tissue morphogenesis by maintaining undifferentiated cells. *HES1* encodes a basic helix loop helix (bHLH) transcriptional repressor that functionally antagonizes positive bHLH genes, such as the endocrine determination gene neurogenin-3 (*NGN3*)[71]. We generated a new Tg pig model for diabetes through genetic engineering of the *PDX1* and *HES1* genes[72,73]. We confirmed pancreatic agenesis in *PDX1-HES1* Tg mid-gestation fetuses and established primary fibroblast cultures from Tg fetuses for somatic cell-based cloning. The cloned fetuses and offspring showed pancreatic agenesis. For the production of Tg porcine strain, we used the blastocyst complementation technique, as described elsewhere[74]. Subsequently, a Tg chimera pig with germ cells carrying a construct expressing *HES1* under the control of the *PDX1* promoter was mated with wild-type (WT) gilts to obtain Tg piglets. These Tg piglets had a high rate of perinatal death due to severe diabetes, although this phenotype could be rescued by insulin treatment. β-cells were not detected, even in the adult pancreas, although other endocrine cells were detected, and exocrine cells functioned normally. The pigs showed no abnormalities in any organ, except for diabetes-associated pathological alterations, such as retinopathy and renal damage. *PDX1-HES1* Tg pigs showed the induction of a stable diabetic phenotype and manifested diabetes-associated complications relatively early. The Tg pig recapitulated several phenotypic manifestations of DM. Therefore, this model seems useful for elucidating the underlying causes and for developing novel treatments for diabetic retinopathy and nephropathy as well as diabetes-related complications.

Apancreatic *PDX1*-KO phenotype generated by genome-editing: We generated diabetic Tg pigs using different strategies[67,72,73]: Tissue-specific and developmental stage-specific hyperexpression of specific genes and KO of master regulator gene. Porcine male fetal fibroblast cells carrying transcription activator-like effector nuclease (TALEN)-induced biallelic mutations in exon 1 of *PDX1* were used for SCNT. Cloned embryos were generated from nuclear donor cells and transferred to recipient gilts. Analysis of cloned fetuses retrieved at mid-gestation (day 55) revealed that *PDX1*-KO mutations generated an apancreatic phenotype[62].

GENERATION OF OTHER TG PIGS IN OUR LABORATORY FOR DIABETIC RESEARCH

The other Tg pig models we generated may play an important role in finding answers to the issues in β-cell replacement therapy for T1DM (Table 1).

Tg pigs with pancreas-specific expression of green fluorescent protein

Genetically modified pigs that express fluorescent proteins, such as green and red fluorescent proteins, have become indispensable in biomedical research. The *PDX1* gene promoter is conjugated to Venus, a green fluorescent protein, and then introduced into 370 *in vitro*-matured porcine oocytes by ICSI-MGT. From these Tg pigs, a Tg-chimeric boar that produced fertile sperm carrying the *PDX1-Venus* expressing vector was obtained[74]. After confirming specific Venus expression in β-cells, the Tg-chimeric boars were mated with WT gilts to acquire Tg piglets that exhibiting green fluorescence in β-cells. These Tg pigs were used in our basic diabetic research.

Generation of two Tg cloned porcine models expressing the far-red fluorescent protein Plum and modified Plum

Monomeric Plum, a far-red fluorescent protein with photostability and photopermeability, is potentially suitable for *in vivo* imaging and detection of fluorescence in body tissues. Using the same technique applied in Tg pig to express green fluorescence in β-cells, we generated Tg cloned pigs that exhibit systemic expression of Plum using SCNT technology. Nuclear donor cells for SCNT were obtained by introducing a Plum-expression vector driven by a combination of the cytomegalovirus early enhancer and chicken β-actin promoter into porcine fetal fibroblasts (PFFs). These Tg pigs exhibited high levels of Plum fluorescence in the skin, heart, kidney, pancreas, liver, spleen, blood cells, lymphocytes, monocytes, and granulocytes[75]. Cell or tissue

transplantation studies using fluorescent markers should be conducted to ascertain whether the xeno-antigenicity of the fluorescent proteins affects engraftment or graft survival. Therefore, we generated a Tg-cloned pig harboring a derivative of Plum modified by a single amino acid substitution in the chromophore. The cells and tissues of this Tg-cloned pig expressing the modified Plum (mPlum) did not fluoresce. However, Western blotting and immunohisto-chemical analyses clearly showed that mPlum had the same antigenicity as Plum. Thus, we have obtained primary proof of principle for creating a cloned pig that is immunologically tolerant to fluorescent protein antigens[76,77]. Transplantation between these two pigs provides much information on aspects such as appropriate transplantation site, behavior of the transplanted organ, and/or cells without any influence of xeno-antigenicity.

Production of medical-grade pigs using the uterectomy-isolated rearing method

The domestic pig best meets the criteria for xenotransplantation. The establishment of an efficient system for the production of designated pathogen-free (DPF) pigs is a prerequisite for the clinical application of xeno-islet transplantation therapy. Therefore, we developed a feasible and economic method that consists of uterectomy of a full-term sow, recovery of fetuses from the uterus, and rearing of neonatal piglets under aseptic conditions in specially designed isolator units. Full-term sows were subjected to a midventral incision under general anesthesia. Each excised uterus was transferred to the recovery unit through a disinfecting tube, and the fetuses were recovered from the uterus. The piglets thus recovered were then transferred to the second isolator unit for artificial nursing with a γ-irradiated milk substitute. Swab samples of the body surface of the pigs and the internal surface of the rearing unit were collected weekly to test the sterility of the piglets. The samples were examined using a standard protocol that was developed and recommended by the Japanese Association for Laboratory Animal Science. At the end of the rearing period, the blood and internal organs, including the pancreas, were collected to analyze sterility and the presence of viruses. The average recovery rate of live piglets was 93.3%. The body weight of the neonatal piglets did not differ significantly from that of the control piglets that were obtained *via* natural farrowing. Blood and tissue samples obtained from the piglets after the rearing period tested negative for bacteria, fungi, and protozoa. Of the viruses currently designated in the guideline for xenotransplantation by the Japanese Ministry of Health, Labor and Welfare, 58 were proven to be absent based on the results of negative polymerase chain reaction analysis. The uterectomy-isolated rearing method has potential applicability for the practical production of donors using neonatal piglets for xeno-islet transplantation[78].

Generation of α1,3-galactosyltransferase and cytidine monophospho-N-acetylneuraminic acid hydroxylase gene double-KO pigs

Target editing is possible through site-specific nucleases, of which the following are most commonly used: zinc finger nucleases (ZFN) and TALEN systems. Pigs can be modified to create porcine models of human disease[35] or provide tissue and organs for xenotransplantation[44]. Thus far, our team has knocked out α1,3-galactosyltransferase (*a1,3GT* or *GGTA1*) and cytidine monophospho-N-acetylneuraminic acid hydroxylase (*CMAH*) genes in pig using genome editing technology and SCNT[79]. Porcine fibroblast cell lines were derived from the *GGTA1*-KO pigs. These cells were subjected to an additional KO of *CMAH* gene. A pair of ZFN-encoding mRNAs targeting exon 8 of the *CMAH* gene was used to generate heterozygous *CMAH*-KO cells, from which cloned pigs were produced by SCNT. One of the cloned pigs was re-cloned after additional KO of the remaining *CMAH* allele using the same ZFN-encoding mRNA to generate *GGTA1/CMAH*-double homozygous KO pigs. The use of TALEN-encoding mRNAs targeting exon 7 of the *CMAH* gene resulted in efficient generation of homozygous *CMAH*-KO cells, which were used for SCNT to produce cloned pigs that were homozygous for a double *GGTA1/CMAH* KO. The combination of TALEN-encoding mRNA, *in vitro* selection of the nuclear donor cells, and SCNT provides a robust method for generating KO pigs. ZFN and TALEN are new tools for producing gene-KO animals. In this study, we produced genetically modified pigs in which two endogenous genes were knocked out. Porcine fibroblast cell lines were derived from homozygous α1,3-galactosyltransferase (*GalT*) KO pigs. These cells were subjected to an additional KO for the cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (*CMAH*) gene. A pair of ZFN-encoding mRNAs targeting exon 8 of the *CMAH* gene was used to generate heterozygous *CMAH*-KO cells, from which cloned pigs were produced by SCNT. Subsequently, one of the cloned pigs was re-cloned after additional KO of the remaining *CMAH* allele using the same ZFN-encoding mRNA to

generate *GalT*/*CMAH*-double homozygous KO pigs. The use of TALEN-encoding mRNAs targeting exon 7 of the *CMAH* gene efficiently generated homozygous *CMAH*-KO cells that were used for SCNT to produce cloned pigs that were homozygous for a double *GalT*/*CMAH*-KO. These results demonstrate that the combination of TALEN-encoding mRNA, *in vitro* selection of nuclear donor cells, and SCNT constitutes a robust method for generating KO pigs.

Production of mini-pig models exhibiting phenotypes resembling those of human diabetes

We previously generated Tg cloned diabetic pigs introduced by dominant-negative mutant *HNF1a*[67]. However, rearing pigs is expensive and technically difficult. As micro-mini pig handling is easier than managing common domestic pigs, we produced Tg pigs using gamete intrafallopian transfer (GIFT), a tool used for assisted reproductive technology against infertility in humans. In vitro fertilization and intrafallopian insemination in the micro-mini pig using the cryopreserved epididymal sperm of a Tg-cloned pig carrying a dominant-negative *HNF1a* gene resulted in a diabetes model with smaller Tg offspring compared to the original model. Thus, relative body weight of the Tg-mini-pig was markedly reduced, reaching 50% that of domestic pigs at 12 wk of age (Tg-mini-pig *vs* WT, 12 kg *vs* 24.6 ± 2.7 kg, Figure 1). The Tg mini-pig developed diabetes-associated complications, such as hyperglycemia and cataract, similar to those of human DM patients, and nodular lesions in the renal glomeruli, similar to that in the original Tg pigs. Thus, artificial reproductive technology using cryopreserved epididymal sperm and GIFT is a practical option for generating Tg mini-pigs.

Production of PDX1-HES1 Tg SCID-pig models receptive to human cells

So far, we have generated interleukin-2 receptor gamma (*IL2RG*) KO pigs *via* the SCNT method in a short duration[80]. The combination of ZFN-encoding mRNAs and SCNT provides a simple and robust method for producing KO pigs without genomic integration. The *IL2RG* gene was knocked out in PFFs using ZFN-encoding mRNAs, and *IL2RG*-KO pigs were subsequently generated using these KO cells through SCNT. The resulting *IL2RG*-KO pigs completely lacked a thymus and were deficient in T and NK cells, similar to that in human patients with X-linked SCID. By mating this pig with *PDX1-HES1* Tg pig[72,73], we generated an SCID pig with pancreatic agenesis that can greatly contribute to preclinical evaluations of stem cell transplantation and xenotransplantation.

SEEKING ANSWERS FOR β-CELL REPLACEMENT THERAPY IN THE CLINICAL SETTING

Our group focuses on diabetes treatment through β-cell replacement therapy, especially cell transplantation. The issues of β-cell replacement therapy in the clinical setting requires several factors to be clarified (Table 1). The issues and challenges are described as follows.

Shortage of donors: Alternative islet cell sources

The number of patients awaiting transplantation is constantly increasing. Moreover, the available organ donor supply will remain insufficient to match the potential demand if cellular replacement therapies play a greater role in the treatment of T1DM patients. The donor islets available are only adequate to treat 1%-2% of potential transplant recipients[75]. In addition, most cases require two to three donor organs[31]. Thus, alternative strategies, including gene therapy, stem cell transplantation, and xenotransplantation, are being explored to bridge this gap.

Gene therapy: Trans-differentiation of cells from a patient's own tissue into pancreatic β-cells could address one of the challenges of donor shortage. The advantage of this strategy is that the patient does not require immunosuppressive drugs. Transfecting non-islet cells to contain and express glucose-regulated insulin is an attractive approach, and the studies have proved challenging[81-83]. However, the efficiency was extremely low, even in mice, and the use of viruses poses some limitations in clinical use.

Stem cell transplantation: Technologies in the field of regenerative medicine provide enormous opportunities for generating β-cells from different stem cell sources for

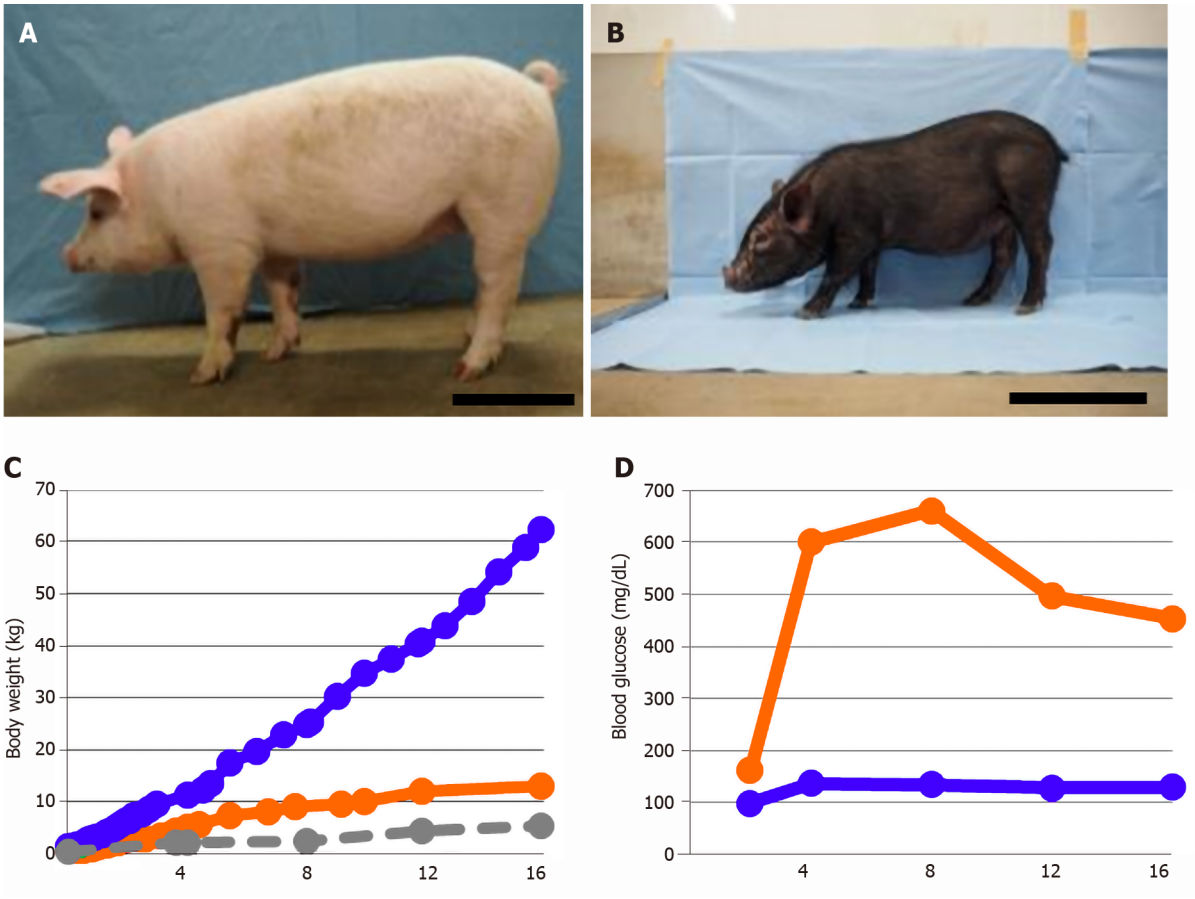

Figure 1 Generation of transgenic-mini pigs carrying a dominant-negative mutant *HNF1α* gene. A: Common domestic pig; B: Transgenic (Tg)-mini pigs carrying a dominant-negative mutant *HNF1α* gene; C: Body weights at specified times; D: Blood glucose level. Blue line: Common domestic pigs, *n* = 2-4. Orange line: Tg-mini pig, *n* = 1. Gray line: micro-mini pig, *n* = 2-4. Quantitative data are presented as means. Scale bar: 30 cm. Time after birth is indicated in days.

cellular therapy. Insulin-producing cells can be generated from a variety of stem cell types, such as pluripotent stem cells, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs), although ideally, functional cells should be expanded to considerable levels by non-integrative culture techniques.

Mesenchymal stem and bone marrow cells: The ease of isolation, plasticity, and clinical translation to generate autologous cells, mesenchymal stem cells, and bone marrow cells are superior. These cells offer the hope that a personalized cell could be transplanted without the risk of alloimmunity, thereby securing sufficient supply to meet future global requirements[84]. Mesenchymal stem cells and bone marrow cells appear attractive for cell therapy; however, they are not readily converted into functionally mature β-cells[85].

ESCs and iPSCs: Human pluripotent stem cells, such as ESCs and iPSCs, provide unprecedented opportunities for developing cell therapies against diabetes and other diseases. Both ESCs and iPSCs have been used in clinical trials.

Human ESCs (hESCs) have been intensively investigated for their ability to differentiate into pancreatic endoderm cells[86-89]. After the hESCs were successfully differentiated into insulin-producing cells, some clinical trials have been conducted, although without positive results[90]. Despite marked advances in the production of insulin-producing cells from hESCs, important challenges remain. Potential ethical and religious considerations emerge when hESCs are used, as the starting cell population is derived from discarded human embryos. Moreover, hESCs need protection against both allo- and autoimmune rejection in the case of transplantation.

In 2006, Takahashi and Yamanaka[91] developed a protocol for dedifferentiating and transdifferentiating murine fibroblast-derived iPSCs. In the subsequent year, the same group generated adult human fibroblasts-derived iPSCs by using defined factors[92]. In diabetes research, patient-specific autologous iPSC-derived β-cell products can prevent immune rejection and be immunologically compatible. Many researchers have

attempted to generate functional β-cells from human iPSCs (hiPSCs); however, transplanting only β-cells might prove insufficient for treating T1DM. For the appropriate regulation of glucose levels, α-cells are essential[93]. In this regard, Veres *et al*[94] reported significant progress in generating hiPSC-derived pancreatic cells, mainly β-like cells, α-like cells, enterochromaffin-like cells, and non-endocrine cells, using a stem cell-derived-β differentiation protocol. This recent finding can potentially inform the production of a defined and safe therapeutic product, with the removal of proliferative progenitor cells to avoid the risk of tumors[94]. The results of the abovementioned study accelerate hiPSC-derived β-cell replacement in the clinic. Despite this promising approach, iPSC-derived β-cell replacement has several hurdles to cross. Manufacturing patient-specific products constitutes a huge advance although the costs associated with the application of good manufacturing practices in individualized stem cell therapy are immense. In other words, this therapy will require optimization of the differentiation conditions for every batch of iPSCs, and the added substantial costs and operational burden with the process could be astronomical[95].

Pig-to-human xenotransplantation

Xenotransplantation would provide an unlimited and predictable donor source of organs and enable careful surgical planning. Furthermore, the recent progress of porcine organ graft survival in nonhuman primates may spur the possibility of successful xenotransplantation[96]. Pigs have been considered a source of islet replacement therapy for T1DM for many years[97]. In 1994, in a first-in-human trial, Groth *et al*[98] transplanted fetal pig islets encased within the human kidney capsule into T1DM patients. The study was remarkable as porcine C-peptide was detectable for more than 300 d, without any observable serious side effects, although there was no insulin independence or reduction in exogenous insulin requirement. Encapsulated pig islets, branded as Diabecell®, to treat patients with unstable T1DM have undergone clinical trials in New Zealand and Argentina, and encouraging preliminary results indicate correction of hypoglycemic unawareness and improved a modest reduction in the hemoglobin (HbA1c) levels. However, detectable porcine C-peptide was strikingly absent in these subjects[99-103].

Response to the donor-shortage challenge—preparation of porcine pancreatic islets for transplantation to human recipients: In the current scenario for T1DM patients who are candidates for islet cell transplantation, porcine islets have the advantage of being an alternative islet cell source to resolve the issue of donor shortage. The pig may potentially be an unlimited source of organs for patients with diabetes, although the appropriate age for islet isolation is not known. As juvenile pigs are more easily reared in uncontaminated conditions, we analyzed the distribution of endocrine cell clusters by comprehensively evaluating juvenile porcine pancreatic development to propose an appropriate age cut-off for islet isolation from the juvenile porcine pancreas[43]. We found that the isolation of porcine pancreatic islets approximately 35 d after birth may offer benefits with regard to their xenotransplantation potential. Further research into the optimal timeline for islet isolation is being conducted in DPF pigs generated at our research center.

GRAFT FAILURE DUE TO IMMUNE SYSTEM, METABOLIC PRESSURE, AND OXIDATIVE STRESS BY HYPOXIA OR INFLAMMATION

Alternative transplantation sites

The portal vein is the most frequently used site for clinical human islet transplantation, although it is far from being an ideal site. Approximately 60% of transplanted islets undergo apoptosis within the first week post-transplantation, which is attributed to poor engraftment. The engrafted islets are continuously exposed to the hepatic microenvironment, high glycemic levels, low oxygen tension, toxins, and combined innate immune attack through instant blood-mediated immune response (IBMIR), all of which are toxic to islets and lead to premature islet dysfunction/death. Several factors, such as extracellular matrix components and proinflammatory cytokines due to immune rejection, have been considered as contributory factors in graft failure[104-106]. In addition, poor revascularization of islets combined with severe changes in the gene expression of the transplanted islets contributes to late dysfunction that, together with islet death, contribute to the poor long-term results of the demand for a large number of islets to restore glucose homeostasis. Many patients achieve insulin

independence after a portal vein islet infusion, although most eventually resume insulin injections in the long term because of the abovementioned issues[107,108]. The protection of islets for transplantation can be achieved through the addition of anti-inflammatory agents during islet culture and systemically to the recipient after transplantation. Moreover, the administration of anticoagulants might be another way to resolve the attendant issues. Based on the identified problems with regard to the liver transplantation site, exploring alternative transplantation sites would be another possible approach. In experimental animal models, islet transplantation has been attempted at many alternative sites. To date, the subcutaneous space, the renal subcapsular space, muscle, pancreas, eye chamber, testis, and thymus can function as islet transplantation sites to reverse hyperglycemia in small animal models. Few alternative sites have the potential for clinical translation, and generally, evidence is lacking for post-transplant islet function superior to that following intraportal infusion[109]. The omentum is one such potentially good transplantation site[110-112]. The University of Miami is currently conducting a phase I/II clinical trial that involves laparoscopic transplantation of human allogenic islets coated in autologous plasma onto the wrapped omentum[113].

Challenges in finding alternative transplantation sites: Our team has continued the search for alternative islet transplantation sites that provide a better environment for prolonged function and survival. As described above, two different cloned Tg-pig models that express the far-red fluorescent protein Plum and mPlum have been under consideration in the search for alternative islet transplantation sites[76,77].

Continued autoimmunity and alloimmunity

In pig-to-human xenotransplantation, significant phylogenetic distance results in serious immunological problems after transplantation. Xenografts are rejected by the human immune system, and there are several current challenges. Some mechanisms of rejection have been clarified and, therefore, genetic engineering techniques have been applied to establish pig models that are suitable as donors.

Hyperacute xenograft rejection: Porcine organs that are transplanted into human recipients are immediately rejected because of the so-called hyperacute immunological reaction. Xenograft rejection is mainly caused by the Gal antigen, found on the donor's cell surface, that is synthesized by the enzyme GGTA1 and synthesizes the α1,3-galactose (α1,3Gal) epitopes (Galα1, 3Galβ1, and 4GlcNAc-R). Humans lack both the Gal antigen and the GGTA-1 enzyme but have xenoreactive antibodies directed against the porcine Gal antigen, which leads to the activation of the enzymatic complement cascade in the recipient. An optimal solution for the problem of hyperacute rejection is the inactivation of the gene encoding the GGTA-1 enzyme responsible for the formation of the Gal antigen. In 2001, the first heterozygous *GGTA1* -KO pigs were produced[114] and, 1 year later, the first piglets with two KO alleles of the *GGTA1* gene were born[115]. A series of *GGTA1*-KO pigs has been generated using a genome editing system. Pigs with the *GGTA1/CMAH/β-1,4-N-acetyl-galactosaminyl-transferase 2* triple gene KO were generated using the CRISPR/Cas9 system. Cells from these genetically modified animals exhibited reduced levels of human immunoglobulin (Ig) M and IgG binding, resulting in diminished porcine xenoantigenicity[116].

Challenges to hyperacute xenograft rejection: As described in the introduction to the section on the generation of other Tg pigs in our laboratory for diabetic research, our team has knocked out *GGTA1* and *CMAH* genes in pigs using genome editing technology and SCNT[79]. Genetically modified pigs can potentially be used as a source of cells, tissues, and organs for transplantation into human recipients.

Porcine endogenous retroviruses: The risk of cross-species transmission of porcine endogenous retroviruses (PERV) has impeded the clinical application of this approach. Recently, CRISPR-Cas9 was used to inactivate 62 copies of the PERV pol gene in a porcine cell line and resulted in > 1000-fold reduction in PERV transmission to human cells[117-119]. Using a combination of CRISPR-Cas9 and transposon technologies, Yue *et al*[120] showed that pigs with inactivated PERV can be genetically engineered to eliminate three xenoantigens and to express nine human transgenes that enhance the pigs' immunological compatibility and blood-coagulation compatibility with humans. The engineered pigs exhibited normal physiology, fertility, and germline transmission of 13 genes, and 42 alleles were edited[120]. However, in the first phase 1/2a clinical trial of the xenotransplantation of encapsulated neonatal porcine islets into non-immunosuppressed T1DM patients in New Zealand, despite the use of non-genetically modified animals, there was no confirmation of PERV transmission. Therefore, the risk

of PERV-related complications might be considered low[100,103,121].

Encapsulation technologies to address continued autoimmunity and alloimmunity: The potential to protect transplanted islets or stem cells from immune attack through micro- or macro-encapsulation approaches has been explored extensively. Encapsulation selectively permits passive diffusion of glucose, insulin, oxygen, carbon dioxide, and other nutrient exchange without direct cell–cell contact with immune cells. Encapsulation technology is potentially promising, although several factors, including the site of transplantation, device configuration, materials, nutrient exchange, and their ability to promote neovascularization and biocompatibility, need to be considered when evaluating such devices[122,123]. A small number of islet encapsulation systems have been applied currently in clinical trials; however, there are insufficient data on the efficacy of these systems in humans, their adverse effects, and the duration that the product can remain safely implanted and functional[122,124,125]. Thus far, none of the trials have resulted in the maintenance of an insulin-independent status in T1DM patients[125,126]. Strategies to overcome these hurdles are being investigated, and some of these interventions are in the clinical trial stage, providing hope to improve results in clinical islet transplantation[126,127].

Challenges with the use of encapsulation technologies: Several studies have demonstrated islet cell survival within encapsulation devices and production of insulin in mice, although translation to larger animals or humans is often limited by fibroblastic overgrowth around the classical implanted device[124]. We performed transplantation of encapsulated juvenile isolated islets in adult pigs in a preliminary experiment. The pig islets were alginate encapsulated, which is generally accepted in this research and clinical field; however, the intraabdominally transplanted capsules developed peri-device fibroblastic overgrowth and adhered to the diaphragm, hepatic surface, and peritoneum. Thus, in a clinical setting of islet xenotransplantation, juvenile isolated pig islet encapsulation with some modifications might be the best method because controlling all targets for rejection may be difficult. Our conclusion is that other biocompatible materials for encapsulation as new devices or changes in the islet transplantation methods are required.

IMMUNOSUPPRESIVE DRUGS

Protecting against immunosuppressant-related toxicity

One of the important causes of immunosuppressant-related toxicity is that currently used immunosuppressive drugs confer a risk for allosensitization in patients and are toxic to islets. Immunosuppressive drugs effectively paralyze immune responses to alloantigens as well as increase the risk of life-threatening infections or malignancies. The most potent immunosuppressive drugs (cyclosporine, tacrolimus, and sirolimus) are directly toxic to β-cells[128-130]. The drugs reduce mitochondrial density and function without changing apoptosis rates, resulting in decreased insulin secretion. Thus, post-transplantation diabetes is possibly induced by the drug's action on mitochondrial function[128]. The immunosuppressive drugs used in clinical islet transplantation are uniformly used for all patients and are not tailored in individualized therapy. Therefore, the present islet transplantation approach and future stem cell therapies cannot be considered ideal until such treatments can be maintained without chronic immunosuppression.

Challenges to protecting against immunosuppressant-related toxicity: We developed a new selection system for suitable immunosuppressive drugs for islet transplantation using epigenetic analysis in a three-dimensional culture system[131]. We first isolated islets from a Tg pig specifically expressing the green fluorescent protein in the β-cells (*PDX1-Venus* Tg Pig). The islets were cultured in one well in the presence of an immunosuppressive drug. Thereafter, epigenetic analysis of the islets was performed. Over time, we observed epigenetic changes in the insulin gene promoter of the islets in a certain group, despite no change in their configuration. This proposed system can evaluate the short-term influence of immunosuppressive drugs on pancreatic islets and can potentially be used to determine which combination of immunosuppressive drugs is specifically suitable for each human islet transplantation. The weak point of the system is that the toxicity of β-cells can be evaluated only *in vitro*. However, if T cells and/or antibodies that are influenced by β-cells from T1DM patients are extracted, the system may potentially enable the identification of new drugs for T and

B cell–depleting antibodies.

BEHAVIOR OF THE TRANSPLANTED CELLS IN THE GROWING BODY IN TRANSPLANTATION PERFORMED IN ADOLESCENTS/CHILDREN

The number of islet transplantations has been increasing in an active and flourishing manner over the past decades. Islet transplantation is seldom performed in younger T1DM patients. Much information would be needed for transplantation of islets in younger patients, such as adverse effects on sexual function or fertility. Research using pigs might provide some information about these questions because pigs grow fast.

APPLICATION OF PIGS FOR THE DEVELOPMENT OF NEW THERAPIES FOR DIABETES

As of 2011, according to the Organ Procurement and Transplantation Network, there were approximately 8000 deceased organ donors in the United States; however, only 1562 pancreas were recovered from donors[132]. Many donated pancreases are unsuitable for extracting islets for transplantation because they do not meet the selection criteria. In addition, islet isolation is complicated and technically difficult in some cases. Islets are often damaged or destroyed during processing. Even in leading centers, it is difficult to recover a sufficient number of islets from a single cadaveric donor pancreas. An average islet isolation generally yields approximately 50% of the estimated number, *i.e.*, more than one million islets present in an adult human pancreas[133,134]. Therefore, at present, islets with a low yield at isolation are not transplanted and distributed for basic research use[135]. Thus, only a small number of islet transplantation can be performed each year. The development of an optimal islet cryopreservation method would permit the banking of unlimited islets and allow the transplantation of islets from multiple donors by a single procedure with a more flexible transplantation schedule. A library of cryopreserved islets allows for selection based on human leukocyte antigen (HLA) tissue type and creates more flexibility with regard to the total amount of transplantable mass. Therefore, pancreatic islet sheets may provide a promising option for the cryopreservation of islets. Successful cryopreservation of pancreatic islet sheet function for both pigs and humans could create a huge stock and delivery system that could alleviate islet shortage. The development of islet cryopreservation methods was initiated more than 30 years ago[136,137]; in the early 1990s, cryopreserved islets were used to facilitate the transplantation of an adequate β-cell mass by combining fresh and cryopreserved islets from multiple donors[138]. However, the use of cryopreserved islets has been hindered due to suboptimal recovery of islet quality and quantity[139-142].

Cryopreservation of pancreatic islets

We have established a new cryopreservation method for pancreatic islets by vitrification using hollow fibers as containers[139]. A unique feature of the hollow fiber vitrification (HFV) method is that it achieves stable vitrification using a minimum volume of cryoprotectant (CPA) solution, thereby ensuring high islet viability. The cytotoxicity, optimum composition, and concentration of CPA for vitrifying islets were examined in a mouse study. Insulin secretion was measured *in vitro* by a static incubation assay, and metabolic functions were tested after transplantation into streptozotocin-induced diabetic mice. The combination of 15% dimethyl sulfoxide and +15% ethylene glycol resulted in the best CPA solution for the HFV of islets. HFV showed the highest viability in comparison to the two vitrification methods, open pulled straws, and vitrification with EDT324 solution. The vitrified islets stably expressed the β-cell markers *NeuroD, Pdx-1*, and *V-maf musculoaponeurotic fibrosarcoma oncogene homolog A*. Transplantation of the vitrified islets achieved euglycemia in the host diabetic mice and similar responses to an intraperitoneal glucose tolerance test as in non-vitrified transplanted islets. The HFV method allows for efficient long-term cryopreservation of islets. This method is being currently tested for application in porcine islets to evaluate the possibility of developing this method for human islet transplantation.

Sheet technology

Transplantation of islet cell sheets has been considered as a feasible, safe, and therapeutically effective approach for the treatment of T1DM patients. The cryopreservation of islet sheets is another option to be considered. Islet sheet transplantation is easily feasible using laparoscopy and does not induce concerns about IBMIR. In a preliminary study, we first generated islet cell sheets with mouse islets[143]. The cell sheets were fabricated using temperature-responsive culture dishes. The engineered islet cell sheets were stable, and the *PDX-1* promoter methylation and the expression of NeuroD, PDX-1, and glucagon proteins were similar between the sheets and islets. Moreover, in the transplantation of islet cell sheets, because of their adhesive properties, cell sheets can be easily applied to the liver and peritoneal surfaces. The mouse islet sheet did not correct serum hyperglycemia in diabetic recipient mice in our study, although other researchers have reported that multi-layered cell sheets boost glycemic stability and insulin function[144-146]. Lee *et al*[147] constructed multilayered cell sheets using rat/human islets and human adipose tissue-derived stem cells. We have successfully generated islet sheets from Tg pigs with a pancreas-specific expression of green fluorescent protein (*PDX1-Venus* Tg Pig) and transplanted them onto the liver surface (Figure 2). Currently, the application of multi-layered pig islet cell sheets is being investigated, and, if feasible, they will be cryopreserved and applied in human transplantation. In addition, islet sheets from pigs with macroencapsulation may be another option.

GARGANTUAN PROJECTS

Production of human tissues and organs in living pigs

The technology used for producing cells and/or tissues of human origin in the bodies of living animals is referred to as *in vivo* bioreactors. Based on proof-of-concept studies in small animals used to produce organs[148-154], we have challenged two different approaches to induce human pancreas in pigs by techniques involving early embryos of animals and the injection of human cells into fetuses or neonatal pigs.

Pigs with human pancreas created by blastocyst complementation as the embryonic approach: Yamaguchi *et al*[155] established a developmental engineering method for heterogeneous mice with rat pancreas. In this concept, a master gene of the pancreatic development *PDX1* gene in rats is knocked out. After fertilization, the blastocyst of *PDX1*- KO rats were complemented with mouse iPSCs. Therefore, the lack of the rat pancreas is complemented by the mouse pancreas. Islets taken from the chimeric rats with mouse pancreas produced an effect when transplanted into a diabetes-induced mouse model[155]. For the application of this concept in porcine models and human studies, apancreatic pigs need to be produced. After blastocyst exchange with a healthy pig, a pancreas is formed from the exogenous cells as the apancreatic pig could not survive for a long time[64,72,73]. Using this concept, iPSCs from T1DM patients can be injected into apancreatic pig embryos to generate human organs inside the pig's body. Once the developing embryo is implanted into a surrogate mother pig, the pig fetus has a human pancreas from the developmental stage. Thus, pigs with personalized human pancreas for T1DM patients can be produced. We have proven the complementation concept in pig-to-pig transplantation as the basic research proof of concept in large animals[64,72], and this technology is currently under consideration to create human pancreas in pigs.

Fetal approaches *via* injection of human cells into fetal pig's organs: This concept involves the use of the developmental niche of pig fetuses to promote pancreatic development from exogenous pluripotent cells. To generate an organ from exogenous cells, developmental circumstances within the fetus make it an ideal *in vivo* bioreactors. Crossing the chimeric male boar carrying the *PDX1-HES1* gene (*PDX1-HES1* Tg pig, described above) with a WT female would produce the next generation of fetuses or newborns with pancreatic agenesis. Direct manipulation of *PDX1-HES1* Tg-pig fetuses to fill the organ niche with pancreatic progenitor cells from hiPSCs may possibly generate human pancreas in the niche. As several groups have already generated insulin-producing cells from hiPSCs[93,94,156-160], we are currently conducting experiments in porcine models using pig progenitor cells to determine whether the concept is feasible. The pancreas was harvested from 40-d-old *PDX1-Venus* Tg-pig fetus, minced, and injected, under ultrasonographic guidance, through the uterine wall into the peritoneal cavity of 40-d-old pig fetus with pancreatic agenesis to track the

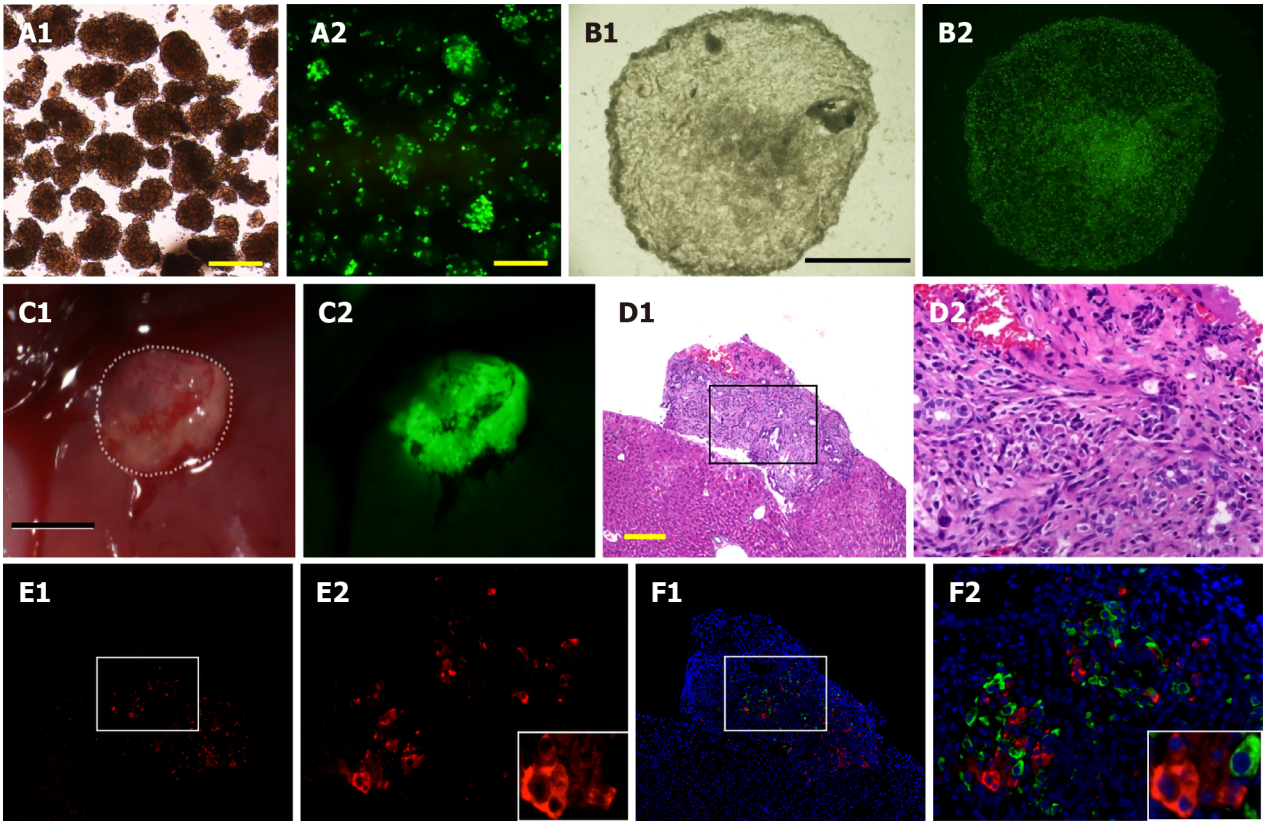

Figure 2 The engineered islet cell sheets. A: Isolated pancreatic islets from a *PDX1-Venus* transgenic pig. Bright-field image of isolated islets (A1). Fluorescence microscopic image of the islets (A2). The *PDX1-Venus* transgenic (Tg) pig expresses green fluorescent protein specifically from the β-cells. The *PDX1* gene promoter was conjugated to *Venus*, a green fluorescent protein. The isolated islets strongly emit green fluorescence in their nuclei; B: Islet cell sheets that were generated *in vitro* by seeding dispersed primary islets cells from a *PDX1-Venus* Tg pig into temperature-responsive 24-well culture plates covered with laminin. Bright-field image of the islet cell sheet (B1). Fluorescence microscopy image of the islet cell sheet (B2); C: The islet cell sheet was harvested 3 d after plating and transplanted onto the liver of streptozotocin-induced diabetic severe combined immunodeficiency mice. Bright-field microscopy. The sheet attached to the liver of diabetic mice (C1). The sheet emitted green fluorescence (C2); D-F: Immunohistochemical analysis of the transplanted islet cell sheet. Hematoxylin–eosin (HE) staining of the sheet on day 23 (D1 and D2). Immunofluorescence analysis of transplanted islet cell sheets (E1, E2, F1 and F2). Glucagon-positive cells (cytoplasm; green) and merged images (insulin-positive cells, cytoplasm; red) (F1 and F2). (D2), (E2), and (F2) present higher magnification images of the region indicated by a square in the panels (D1), (E1), and (F1), respectively. Nuclei were stained blue with DAPI (4,6-diamidino-2-phenylindole). Scale bars: yellow, 200 μm; black, 5 mm. The time after transplantation is indicated in days.

subsequent development of pancreatic tissue (Figure 3). On day 14 after transplantation, the minced-fetal pancreas was attached to the liver and under the diaphragm. The cells survived, and histological analyses confirmed progressive development of ductal structures characteristic of the fetal pancreas[161]. Based on this concept, a chimeric human pancreas may be obtained with a pig scaffold.

CONCLUSION

Appropriate animal models for the evaluation of the efficacy and safety of new therapeutic concepts are critical for the successful application of translational medicine. Genetically tailored porcine models have the potential to bridge the gap between proof-of-concept studies in animals and clinical studies in patients with T1DM. The translation of novel discoveries to clinical applications is a long process that is costly and often inefficient. However, these efforts definitely hold potential for the provision of appropriate treatments within a reasonable time frame. We believe that new therapeutic approaches for solving the issues with β-cell replacement therapy to treat diabetes will be imminently discovered, validated, and optimized in large animal models, which will help save patient lives.

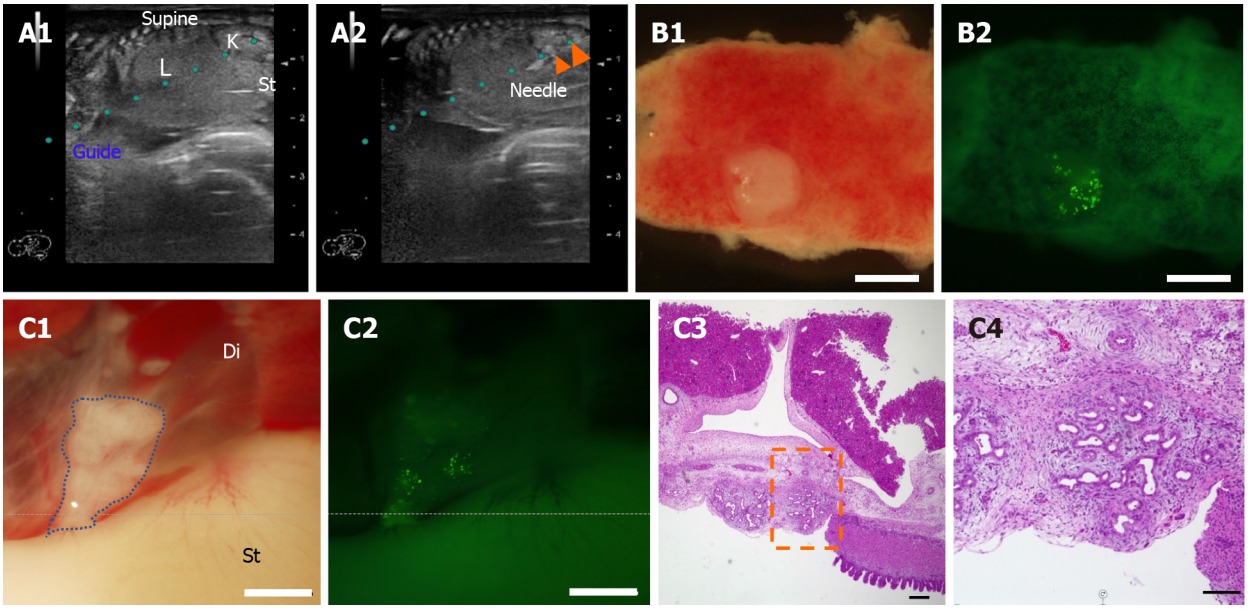

Figure 3 Ultrasound-guided fetal pancreatic tissue injection into a porcine fetus with organ niche. A: The pancreas was harvested from the 40-d-old *PDX1-Venus* transgenic-pig fetus and minced. The minced-fetal pancreas was injected, under ultrasonographic guidance, through the uterine wall into the peritoneal cavity of a 40-d-old pig fetus. Peritoneal cavity of a 40-d-old pig fetus and a guide for the needle (A1). The needle was inserted into the omental foramen, and the minced-fetal pancreas was injected (A2); B: The minced-fetal pancreas was attached to the liver on day 14 (B1) and emitted green fluorescence (B2); C: The minced-fetal pancreas was attached under the diaphragm on day 14 (C1) and emitted green fluorescence (C2). Hematoxylin–eosin staining of the pancreas on day 14 (C3 and C4). The transplanted cells survived, and histological analyses confirmed the progressive development of the pancreatic ductal structures. (C4) comprises a higher magnification image of the region indicated by a black square in panel (C3). The time after birth is indicated in days. Scale bars: white, 1 mm; black, 5 μm. L: Liver; K: Kidney; St: Stomach; Di: Diaphragm.

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REVIEW

Exercise intervention under hypoxic condition as a new therapeutic paradigm for type 2 diabetes mellitus: A narrative review

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Abstract

This review aims to summarize the health benefits of exposure to hypoxic conditions during exercise in patients with type 2 diabetes mellitus (T2DM). Exposure to hypoxic conditions during exercise training positively changes the physiological response in healthy subjects. Exposure to hypoxic conditions during exercise could markedly increase skeletal muscle glucose uptake compared to that in normoxic conditions. Furthermore, post-exercise insulin sensitivity of T2DM patients increases more when exercising under hypoxic than under normoxic conditions. Regular exercise under short-term hypoxic conditions can improve blood glucose control at lower workloads than in normoxic conditions. Additionally, exercise training under short-term hypoxic conditions can maximize weight loss in overweight and obese patients. Previous studies on healthy subjects have reported that regular exercise under hypoxic conditions had a more positive effect on vascular health than exercising under normoxic conditions. However, currently, evidence indicating that exposure to hypoxic conditions could positively affect T2DM patients in the long-term is lacking. Therefore, further evaluations of the beneficial effects of exercise under hypoxic conditions on the human body, considering different cycle lengths, duration of exposures, sessions per day, and the number of days, are necessary. In this review, we conclude that there is evidence that exercise under hypoxic conditions can yield health benefits, which is potentially valuable in terms of clinical care as a new intervention for T2DM patients.

Key Words: Exercise; Hypoxia; Insulin-resistance; Metabolism; Type 2 diabetes mellitus;

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Weight loss

Core Tip: Current research shows that exercise interventions performed under hypoxic conditions have positive effects on healthy subjects and athletes. Exercise intervention under hypoxic conditions can be beneficial as a new treatment for patients, including those with diabetes. This review summarizes recent studies on the potential cause–effect relationship for exercise interventions under hypoxic conditions in type 2 diabetes mellitus patients and discusses health benefits and risk factors.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a severe global public health problem. The worldwide diabetes prevalence in 2019 was estimated to be 463 million people, with a projected increase to 578 million by 2030 and 700 million by 2045[1]. Diabetes is a primary cause of preventable lower limb amputations, blindness, and end-stage renal failure[2]. It is also associated with increased cardiovascular complications and premature death[3].

Insulin is the main hormone produced by pancreatic β-cells in the islets of Langerhans. It reduces blood glucose by stimulating glucose absorption into tissues, including fat and muscles. Insulin action refers to insulin signal cascade activation that is stimulated by insulin binding to its receptor, causing glucose and lipid absorption and metabolism, gene expression and protein synthesis, cell growth, and survival[4]. Skeletal muscle is the leading glucose-uptake site[5], one of the principal organs of insulin action, and is involved in glucose homeostasis regulation in healthy and diabetic conditions[6]. In hyperinsulinemia, uptake of insulin-mediated blood glucose in skeletal muscle accounts for about 95% of whole-body-based glucose disposal during hyperglycemia[5]. Glucose requires specific transfer proteins to be transported across the cell membrane[7].

Glucose transporter 4 (GLUT4) is primarily responsible for this action[8-10]. GLUT4 is the primary transporter of glucose in skeletal muscle[9]. Increased GLUT4 expression in the skeletal muscle membrane is an essential indicator of exercise-related improvement[11]. The carrying rate of glucose in skeletal muscle is the limiting stage for glucose uptake at rest, and the translation and expression of GLUT4 in response to exercise determine the acute regulation of glucose uptake[11]. Previous studies have reported impaired insulin-stimulated glucose uptake and a decreased rate of glycogen synthesis in insulin-resistant muscles[12,13]. The reduction of glycogen synthesis due to insulin-stimulated glucose transport disorder plays an important role in muscle insulin-resistance development[14]. Therefore, skeletal muscle insulin-resistance has been regarded as a significant defect in T2DM[12]. The precise mechanism underlying insulin-resistance in skeletal muscle has not yet been fully elucidated[15]. According to previous reports, the decrease in insulin-stimulated glucose uptake in skeletal muscle is caused by the degradation of insulin signals and a defect in multicellular cascades, such as glucose transport inhibition, glucose phosphorylation, and the reduction of glucose-oxidizing glycogen synthesis, which plays a decisive role in the development of insulin-resistance in skeletal muscle[16]. Thus, it has been reported that the decrease in insulin action in insulin-resistant skeletal muscle is related to the decrease in glycogen synthesis, caused by insulin-stimulated glucose transport disorder and decreased mitochondrial oxidation related to physical inactivity[12,17,18].

It has been established that exercise intervention can enhance insulin action and glycemic regulation ability in individuals with T2DM, which may be due to the increased oxidation capacity of skeletal muscle, resulting in improved β-cell

function[19-22]. Patients with T2DM do not release enough insulin to control glucose due to loss of cells, deterioration of their function, or both[23]. Obesity increases the risk of T2DM[24,25] partly by decreasing insulin sensitivity, *i.e.*, insulin does not cause a normal reduction in blood glucose.

Given the relationship between diabetes, increased cardiovascular disease, and decreased life expectancy[26], the concept of effective treatment for diabetes is important. Exercise interventions promise to enhance glycemic control and cardiovascular condition[19]. It is important to find a more effective strategy for treating this metabolic disease[27]. Recently, many studies have investigated treatment of diabetes by widely applying hypoxic conditions, based on studies that showed that exercising at high altitudes reduced the risk of diabetes, cardiovascular disease, and obesity-related diseases, as compared to exercising at sea level[27-31]. Hypoxic therapy is a novel treatment used as a common practice in many developed countries[28,32-35]. Hypoxic therapies such as hypoxic exposure or hypoxic exercise intervention have been recommended to treat and prevent diabetes by affecting glucose uptake, insulin sensitivity, and vascular health[27].

This narrative review aims to summarize any possible benefits of exposure and exercise under artificially generated hypoxic conditions in T2DM patients.

EFFECT OF HYPOXIC EXPOSURE ON PHYSIOLOGICAL RESPONSES

Short- or/and long-term exposure to hypoxic conditions causes extensive physiological changes[36]. Normobaric (*i.e.*, simulated altitude) and hypobaric hypoxia (*i.e.*, real and simulated altitude) can reduce oxygen partial pressure in tissues and in blood. The acute compensatory response activates the sympathetic nerves and increases ventilation, and causes an altitude-dependent increased cardiac output upon exposure of inhabitants of low-lying areas to high altitudes[37]. Hyperventilation is one of the essential processes involved in supplying sufficient oxygen to tissues[38]. Moreover, peripheral chemical receptors in the carotid body react to reduced arterial partial pressure of oxygen[39]. When a decrease in arterial oxygen saturation is detected by these receptors, the signal leads to sympathetic nerve activation and stimulation of ventilation, increasing the metabolic demand[40]. Exposure to dry and cold environments may increase water loss as ventilation increases[41]. As such, ventilation and cardiovascular reactions ensure that tissues' metabolic demands are met at rest and during exercise at high altitudes. Sustained exposure to hypoxia results in a reduced cardiac output to a similar level as under normoxia. These adaptive responses are facilitated by an increased stimulated red cell mass and further increases in ventilator responses to hypoxia[36].

Intermittent hypoxia intervention has been studied over the past decades as a means of treatment for various health conditions[42-44]. It has been suggested that exposure to mild hypoxia for 1 h, with or without simultaneous exercise, had an acute effect on insulin-resistance and blood glucose level in T2DM patients[45,46]. In addition to these adaptations, exposure to the hypoxic environment positively affected body composition (*e.g.*, fat mass, percent body fat, and fat-free mass)[28,30]. It has also been proven that several weeks of exercise under moderate hypoxia resulted in more weight loss in obese individuals than did exercising at the same or higher intensity under normoxia[29,30,47,48]. Previous studies have also reported the beneficial effects of repeated exposure to intermittent hypoxic interventions for a few weeks, in the absence of other types of intervention, on fasting blood glucose and insulin levels[49,50]. However, the fundamental mechanism underlying changes in glycemic control and insulin sensitivity due to hypoxia have been unclear[51]. In addition to the insulin-dependent regulatory mechanism, it has been speculated that hypoxia may affect glucose uptake in a way similar to exercise[51]. Therefore, exposure to hypoxic conditions is a new method of intervention for health-promotion and prevention or treatment of chronic diseases by improving body weight, cell metabolism, cardiovascular, and respiratory function (Figure 1)[32,52].

HYPOXIC THERAPY FOR T2DM AND ITS APPLICATIONS

Review and meta-analysis suggest that exposure to hypoxic conditions during exercise can improve insulin sensitivity and enhance cardiovascular health more than exposure to normoxic conditions[28,53-55]. Furthermore, exposure to hypoxic conditions has been shown to increase endurance performance in athletes[56-58]. Exercising under hypoxic

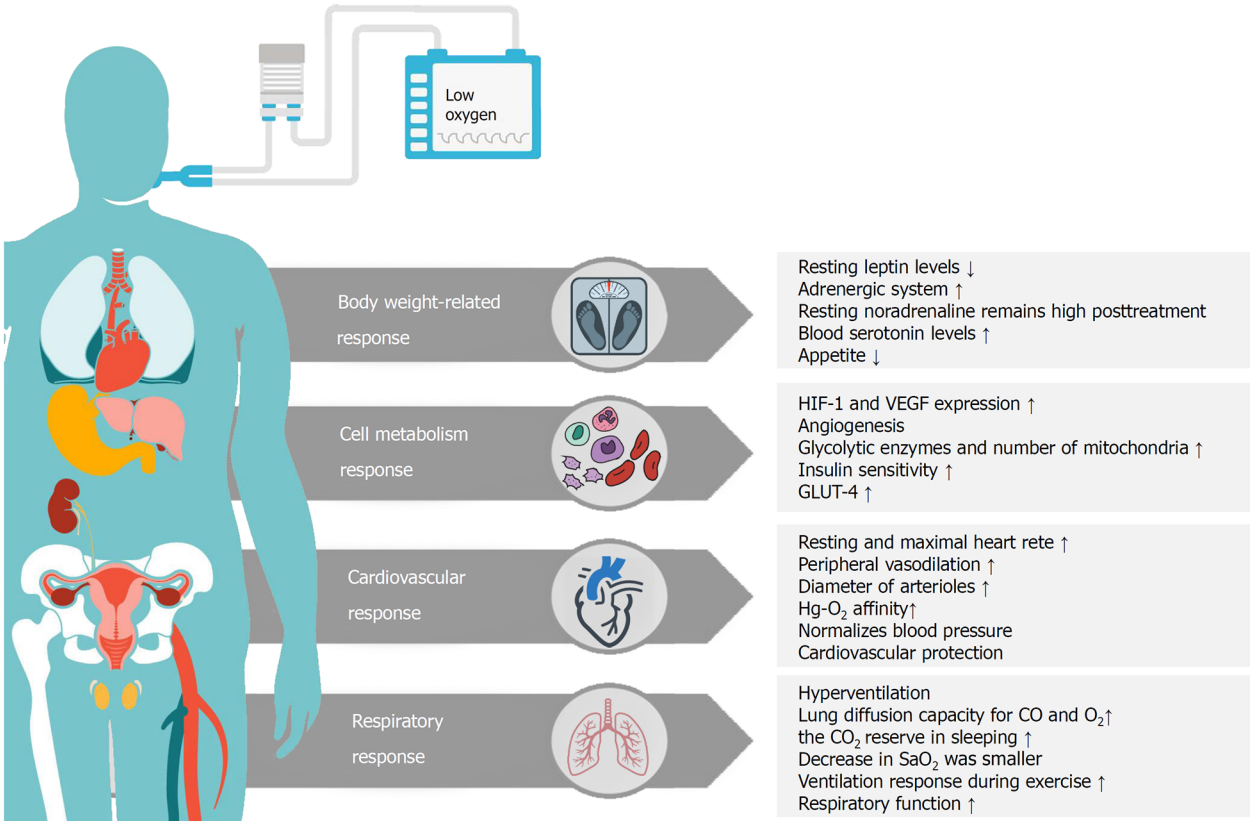

Figure 1 Effect of hypoxic exposure on the physiological response to exercise. Exposure to hypoxic conditions is a new intervention method for health-promotion and for preventing or treating chronic diseases by improving body weight, cellular metabolism, and cardiovascular and respiratory function. HIF-1: Hypoxic inducible factor-1; VEGF: Vascular endothelial growth factor; GLUT-4: Glucose transporter-4; Hg: Hemoglobin; O_2: Oxygen; CO: Carbon monoxide; C_2: Carbon dioxide; Sa_2: Arterial oxygen saturation.

conditions can enhance the exercise adaptations and exercise tolerance of T2DM patients[27]. Previous studies of exposure to hypoxia during acute and chronic exercise in T2DM and insulin-resistant patients are summarized in detail in Tables 1 and 2.

To date, various technical equipment has been developed to create hypoxic conditions artificially. Artificially produced hypoxia can be obtained by changing barometric pressure (hypobaric hypoxia) or by changing the fraction of oxygen (FiO_2) (normobaric hypoxia). FiO_2 is always constant at sea level (ca. 21%), and barometric pressure decreases with higher altitude. Hypoxic conditions can be created by using a special chamber at rest or during exercise. Such an environment control chamber is shown in Figure 2.

Therapeutic effects of exercise intervention under hypoxic conditions on T2DM

Effects of exercise intervention under hypoxic conditions on glucose uptake and insulin sensitivity: Exposure to hypoxia increases glucose uptake in the skeletal muscles of healthy and obese adults[59]. Brooks *et al*[60] demonstrated that 3 wk of hypoxic exposure at an altitude of ca. 4300 m improves glucose turnover and decreases blood glucose in healthy males. Lippl *et al*[61] showed that short-term hypoxic exposure (1 wk at an altitude of ca. 2650 m) decreased glycated hemoglobin (HbA1c) levels in obese males. Exposure to hypoxia can markedly improve glucose uptake, and exposure continuously improves blood glucose regulation over the long term.

A previous review article reported that regular physical activity and exercise could markedly increase peripheral glucose uptake and improve blood glucose regulation[62]. It is necessary to clarify whether exercise and hypoxia can have positive combined effects in T2DM and whether patients with this condition can benefit from short- or long-term exposure to hypoxia during exercise. Mackenzie *et al*[63] examined short-term hypoxic exposure during acute exercise on the glucose homeostasis in T2DM patients. They proved that glucose loss and sustained glucose infusion during cycling under hypoxic conditions were more significantly increased than under normoxic conditions[46,63]. Glucose tolerance tests performed immediately after cycling showed that blood glucose regulation improved more under hypoxic conditions.

Table 1 Effects of acute exercise under hypoxia *vs* under normoxia in patients with type 2 diabetes mellitus

Ref.	Participants	Design and protocol	Exercise intensity	Main results
Mackenzie *et al*[63] (2011)[1]	*n* = 8; sex: Male; age: 58 ± 4.0 yr; BMI: 29.2 ± 6.7 kg/m^2	(1) 60 min rest in normoxia; (2) 60 min rest in hypoxia normobaric hypoxia (FiO_2: 14.6%, simulated altitude: ca.3100 m); (3) 60 min cycling in normoxia; and (4) 60 min cycling in hypoxia (normobaric hypoxia: FiO_2: 14.6%)	(3) and (4): 90% lactate threshold	Blood lactate: ↔ (1), (2); ↑ (3), (4). Blood glucose: ↔ (1); ↓ (2), (3), (4). Insulin sensitivity (during glucose tolerance test): (4) > (3) > (2) > (1)
Mackenzie *et al*[46] (2012)[1]	*n* = 8; sex: Male; age: 58.7 ± 2.2 yr; BMI: 28.3 ± 2.1 kg/m^2	(1) 60 min continuous cycling in hypoxia (normobaric hypoxia: FiO_2: 14.7%, simulated altitude: ca.3100 m); (2) 60 min interval training with passive recovery (5:5 min) in hypoxia (normobaric hypoxia: FiO_2: 14.7%); and (3) 60 min interval training with passive recovery (5:5 min) in normoxia	(1): 90% lactate threshold; (2): 120% lactate threshold; (3): 120% lactate threshold	HR and blood lactate: ↑ (1), (2), (3). Blood glucose decrease (pre- to post-exercise): (1) > (2). Glucose disappearance: ↑ (1); ↔ (2), (3). HOMA-IR index improved after 24 h: ↑ (1), (2); ↔ (3); after 48 h: ↑ (1); ↔ (3)
Brinkmann *et al*[76] (2017)[2]	*n* = 8; sex: Male; age: 58.0 ± 15.0 yr; BMI: 33.0 ± 6.0 kg/m^2	40 min cycling: (1) Normoxia; (2) Hypoxia (normobaric hypoxia: FiO_2: 14%, simulated altitude: ca. 3400 m); and (3) Hypoxia (normobaric hypoxia: FiO_2: 14%) + hyperoxia (normobaric hyperoxia: FiO_2: 30%) intervals (5:5 min)	Blood lactate: 2.5 mmol/L	Blood lactate (post-exercise lower): (3) > (2). BORG RPE: ↔ (1), (2), (3). Pro-angiogenic factors: VEGF: ↑ (2), (3). Anti-angiogenic factor: endostatin: ↑ (2), (3)

[1]Data are presented as mean ± SE.

[2]Data are presented as mean ± SD. BMI: Body mass index; HR: Heart rate; BORG RPE: BORG rating of perceived exertion; FiO_2: Fraction of inspired oxygen; HOMA-IR: Homeostatic model assessment for the quantification of insulin-resistance; VEGF: Vascular endothelial growth factor.

Table 2 Effects of hypoxia *vs* normoxia chronic exercise in patients with type 2 diabetes mellitus or insulin-resistance

Ref.	Participants	Intervention	Intensity	Frequency and duration	Main results
Wiesner *et al*[69] (2010)[1]	*n* = 45. NTG: sex: 8 male, 13 females; age: 42.1 ± 1.7yr; BMI: 32.5 ± 0.8. HTG: sex: 10 male, 14 females; age: 42.2 ± 1.2 yr; BMI: 33.1 ± 0.3	60 min running on a treadmill; normobaric hypoxia: simulated altitude: ca. 2740 m	VO_{2peak}: 65%	3 d/wk, 4 wk	Lactate levels at the anaerobic threshold: ↓ HTG; fasting insulin, HOMA-IR: ↓ NTG, HTG; body fat decreased: HTG > NTG; BP, LDL-c: ↔ NTG, HTG
Schreuder *et al*[66] (2014)[2]	*n* = 19. NTG: sex: 5 male, 4 females; age: 52.0 ± 8.0 yr; BMI: 36.0 ± 6.5 kg/m^2. HTG: sex: 9 male, 1 female; age: 57.0 ± 6.0 yr; BMI: 30.9 ± 4.1 kg/m^2	45 min endurance training (cycling) + series of strength training exercises; normobaric hypoxia: FiO_2: 16.5%: simulated altitude: ca. 2100 m	HRR: 70%-75%	3 d/wk, 8 wk	VO_{2max}: ↑ NTG, HTG; BMI, BP, HOMA-IR, HDL-c, LDL-c, TC, TG, fasting glucose, HbA1c: ↔ NTG, HTG; Vasodilatory function: ↔ NTG, HTG

[1]Data are presented as mean ± SE.

[2]Data are presented as mean ± SD. NTG: Normoxia training group; HTG: Hypoxia training group; BMI: Body mass index; Fi_2: Fraction of inspired oxygen; HDL-c: High-density lipoprotein cholesterol; HbA1c: Glycated hemoglobin; HOMA-IR: Homeostatic model assessment for the quantification of insulin-resistance; HRR: Heart rate reserve; LDL-c: Low-density lipoprotein cholesterol; VO_2peak: Peak oxygen uptake; VO_2max: Maximal oxygen uptake; BP: Blood pressure; TC: Total cholesterol; TG: Triglyceride.

Insulin sensitivity increased only after 24 h and 48 h after exercise under hypoxic conditions. It also reported that exercising at a continuous submaximal intensity under hypoxic conditions effectively increased glucose uptake and insulin sensitivity than interval training[46]. Thus, previous research suggests that acute exercise under hypoxic conditions positively affects glucose uptake and insulin sensitivity in T2DM patients. The increase in glucose uptake during hypoxia has been thought to be due to the upregulation in the glycolytic energy pathway, which compensates for decreased energy production by the aerobic system[64]. Katayama *et al*[65] reported that the respiratory exchange ratio was lower during submaximal cycling exercise for 30 min at sea levels in healthy males than similar cycling under hypoxic conditions (at an altitude of ca. 2000 m). However, Schreuder *et al*[66] found no change in insulin sensitivity and blood glucose regulation in T2DM patients after exercise training under normoxia or short-term hypoxia.

Previous studies were also conducted on insulin-resistance in healthy adults and T2DM patients. Haufe *et al*[67] reported significant improvements in the values for the homeostatic model assessment of insulin-resistance (HOMA-IR) index in healthy males, only during exercise under hypoxic conditions. However, Lecoultre *et al*[68] showed increased glucose and insulin concentrations and higher insulin-to-glucagon

Figure 2 Environment control chamber. Various technical equipment has been developed to create hypoxic conditions. Artificially produced hypoxia can be obtained by changing barometric pressure (hypobaric hypoxia) or by changing the fraction of oxygen (FiO_2) (normobaric hypoxia). The FiO_2 is always constant at sea level (FiO_2 ca. 21%), and the barometric pressure decreases with higher altitude. The hypoxic conditions can be created using a special chamber at rest or during exercise.

rates after exercise training under hypoxic conditions, as compared to under normoxic conditions. Wiesner *et al*[69] showed that exercise training performed under short-term hypoxia did not change the HOMA-IR index in overweight and obese subjects. However, the training workload was significantly lower in the hypoxic group than in the general group, and exercise under hypoxic conditions was a more efficient method. These previous studies also demonstrated improvements in the HOMA-IR index with reduced body fat in the hypoxia groups[67,69]. Decreasing body fat helps to increase insulin sensitivity, particularly in T2DM patients, because there is a positive association between body fat, peripheral insulin-resistance, and pro-inflammatory conditions[70].

Thus, the combination of short-term hypoxia exposure and exercise has a beneficial effect. Nevertheless, there is a lack of biochemical evidence in human research. Therefore, further studies are needed to clarify whether exercise under short-term hypoxia can effectively increase blood glucose uptake and insulin sensitivity, as compared to exercise under normoxia[27].

Effects of exercise intervention under hypoxic conditions on skeletal muscle: The decrease in skeletal muscle capillarization can have a negative effect on blood glucose regulation, and a negative relationship between skeletal muscle capillary density and insulin concentration has been shown previously[71]. Regular physical activity has been shown to have a positive effect skeletal muscle capillaries[53]. Lundby *et al*[72] concluded in a previous review that combining exercise and hypoxia may accelerate structural and functional adaptation. In contrast, prolonged exposure to hypoxia does not result in significant changes in human capillarization during rest[72]. Mizuno *et al*[73] reported that exposure to ca. 5300 m for 75 d did not change the ratio between capillaries and muscle fibers. However, because the reduction in fiber size can be adapted to hypoxic exposure, capillaries per region were increased at a similar altitude[74]. Recent meta-analysis studies have shown that exercise under hypoxic conditions positively affects the skeletal muscle capillaries and function of the vascular dilator[53].

A temporary increase in pro-angiogenic factors due to acute exercise may be related to the initiation and control of angiogenesis[75]. Brinkmann *et al*[76] found that acute exercise under hypoxia could lead to upregulation of serum pro-angiogenic factors, as compared to exercise under normoxia, in T2DM patients. Additionally, there are other

mechanisms that contribute to increased pro-angiogenic regulator release after exercise under hypoxic conditions. First, tissue hypoxia during exercise can be enhanced by environmental hypoxic conditions and is increased by activation of hypoxia-induced factor-1α (HIF-1α), which initiates expression of proteins related to angiogenesis regulation[77]. However, responses to hypoxia in diabetes have been impaired, and hyperglycemia is a very important result in the HIF-1α regulation[78]. The second mechanism is increased sympathetic nerve activity and increased skeletal muscle blood flow and shear stress at the vessel walls, which induce intracellular signal transduction through mechanical stimulation of capillaries, thereby increasing angiogenesis *via* vascular formation[79].

Effects of exercise intervention under hypoxic conditions on vascular health: Exercise can help prevent disease progression and protect T2DM patients from secondary complications by improving long-term increases in skeletal muscle capillarization and vascular function. Patients with diabetes develop not only abnormal angiogenesis but also macroscopic and microscopic angiogenesis with endothelial dysfunction[80]. The current meta-analysis suggested that exercise improves vascular dilation when performed under hypoxia than under normoxia[53]. Exercise under hypoxic conditions is associated with a compensatory increase in blood flow to active muscles to meet the oxygen demand[81,82]. Exercise-induced blood flow is important in inducing vascular adaptation. The combination of exercise and hypoxia can positively affect vascular adaptation in normoxic exercise training, particularly in T2DM patients who typically exhibit attenuated exercise-induced blood flow[83]. However, Schreuder *et al*[66] showed no effect of training on the vascular dilation in T2DM patients, both when exercise was performed in normoxia and hypoxia. These differences may be due to the different training protocols or oxygen concentrations used, and other possible adaptation mechanisms in T2DM patients[84].

The effects of hypoxia and exercise may be related to subsequent increased blood flow to muscles, and high shear stress, nitric oxide (NO), and oxygen tension reduction[81,83,85,86]. While the specific mechanisms that underlie the effects of exercising under hypoxia remain unclear, it has been demonstrated that exposure of endothelial cells to hypoxia increases[87]. In this regard, the expression and activation of endothelial NO synthase (eNOS) as a potent vascular dilator can be increased and produce eNOS and NO levels. Therefore, exercise under hypoxic conditions is thought to have high potential to improve vascular health. However, further studies are needed to confirm that long-term exercise under hypoxia can improve skeletal muscle capillarization more significantly than exercise under normoxia in T2DM patients. Previous studies can also demonstrate how training protocols should be modified to induce effective adaptation of physiological variables. Schreuder *et al*[66] have reported that the vascular dilation in T2DM patients could be positively affected by exercise training under short-term hypoxic conditions. However, additional studies should be conducted to apply various training protocols and oxygen concentrations.

Effects of exercise intervention under hypoxic conditions on body composition: There are positive correlations between increased fat mass, insulin-resistance, chronic inflammation, and cardiovascular disease[70]. Decreasing fat mass is one of the important goals in the treatment of overweight and obese patients with T2DM. Previous studies have reported that exercise under hypoxia can help reduce body weight and body fat mass in overweight and obese patients with T2DM[27,88,89]. Kong *et al*[47] showed that combined aerobics and strength training under hypoxic conditions (simulated altitude: ca. 2100-3200 m, FiO_2: 14.5%-16.5%) for 4 wk (11 sessions/wk) decreased weight more than under normoxic conditions in obese young adults. Wiesner *et al*[69] reported that hypoxia exercise training for 4 wk improved body composition in obese men and women without diabetes and with insulin-resistance. Acute exposure to hypoxia (2 h) at a simulated altitude of 4300 m has been reported to decrease the leptin reaction to glucose uptake in healthy humans[90]. The results of previous studies may be related to changes in hormones that control appetite. However, the effects of acute exposure to hypoxia on leptin levels and appetite in T2DM patients have not been demonstrated. Therefore, a future study on appetite regulation mechanisms is needed, considering that T2DM patients have leptin resistance[91].

Effects of exercise intervention under hypoxic conditions on blood lipids and oxidative stress: Another perspective of the effect of exercise under hypoxic conditions is its effects on blood lipid variables in patients with diabetes. Simpson *et al*[92] examined how exposure to hypoxic conditions during moderate exercise, as well as 16 d of rest in normoxia and continuous normobaric hypoxia (simulated altitude: ca. 3400

m, FiO_2: 14%), changes total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels in healthy men. Exposure to hypoxic conditions during moderate exercise significantly reduced total cholesterol, HDL, and LDL levels. Schreuder *et al*[66] and Wiesner *et al*[69] reported no positive effect of exposure to hypoxic conditions during exercise on blood lipids variables in overweight and obese patients with T2DM and insulin-resistance. To date, there have been no studies providing evidence of an effect of exposure to hypoxic conditions during exercise on blood lipids.

T2DM patients may experience increased oxidative stress as free radicals increase, further exacerbating insulin-resistance or causing cardiovascular disease[93]. However, more research is needed on whether exercise under short-term hypoxic conditions can reduce oxidative stress in T2DM patients and protect against secondary complications caused by free radicals than exercise performed under normoxic conditions. A single bout of interval training under hypoxic conditions (simulated altitude: ca. 4000 m, FiO_2 : 13%) has been reported to increase ventilatory responses in T2DM patients[45]. These results demonstrate the potential of such training to benefit individuals with diabetes with autonomic regulation imbalances. Future research requires verification of the effectiveness of exercise under short-term hypoxic conditions in improving blood lipid levels and oxidative stress.

Possible health risks of exercise intervention under the hypoxic condition on T2DM

This narrative review describes some health risks that may arise when exercising under hypoxic conditions. The definition of the range of oxygen-availability under which exercise can be performed under hypoxic conditions without negatively affecting health needs to be defined. Previous studies have set short-term hypoxic conditions similar to simulated altitudes of up to ca. 4000 m for healthy participants and ca. 3400 m for T2DM patients. These hypoxic conditions did not result in health problems in previous studies. However, breathing air with rapidly reduced oxygen levels or prolonged exposure to very high altitude conditions increases the risk of neurocognitive impairment, myocardial infarction, and stroke[94]. Clinical effects on the human body have not been apparent, but T2DM patients have been shown to respond to hypoxic conditions with low ventilation reactions[95]. A previous study has suggested that cardiac output and heart rate is changed in T2DM patients upon exposure to hypoxic conditions[84]. In particular, T2DM patients with neurological disorders could be negatively affected during exercise involving exposure to hypoxic conditions. In terms of body composition, exposure to long-term hypoxic conditions may facilitate reduction in body weight and fat mass, but exposure to extreme altitude (> 5000 m) has been shown to affect fat-free mass negatively[96]. The effect of increased capillarization in skeletal muscle after exercise training under hypoxic conditions requires further study, and the clinical relevance of excessive abnormal angiogenesis in diabetes needs to be shown[80].

Oxidative stress is exacerbated under hypoxic conditions by both intense and long-term exercise[97]. The actual protocol for exposure to hypoxia has varied significantly across studies in terms of cycle length (*e.g.*, weeks), the duration of exposure (*e.g.*, minutes and hours), the number of exposures per day (*e.g.*, session), and the number of days. Exposure to extreme acute hypoxic conditions may be similar to the findings obtained with animal models of ischemia or reperfusion, with acute release of excessive free radicals and decreased antioxidant capacity[98]. Oxidative stress can cause cell and tissue damage and be harmful to the human body. However, redox balance changes can play a positive role as a potential stimulus for adaptation to prolonged exercise[99]. Previous studies have shown that regular moderate exercise can weaken oxidative stress associated with hypoxia[100-103]. Therefore, there is potential health risks when T2DM patients exercise under hypoxic conditions. However, its value as an effective treatment method would be marked, if appropriate safety precautions are implemented.

CONCLUSION

Short- and long-term exposure to hypoxic conditions during exercise may improve glucose uptake and insulin sensitivity in T2DM patients more than when exercising under normoxic conditions. Additionally, exercising under hypoxic conditions could help reduce body weight and fat mass in overweight and obese patients with T2DM. Several previous studies have reported positive effects of exercise training under

hypoxic conditions on the bodies of T2DM patients. However, there is currently a lack of research on the long-term adverse effects of exposure to hypoxic conditions during exercise training in T2DM patients. Future studies should evaluate the potential benefits of exposure to hypoxic conditions during exercise, to design new intervention methods (normobaric hypoxia *vs* hypobaric hypoxia) for treating T2DM patients. Overall, exposure to hypoxic conditions during exercise in T2DM patients have the potential value of adaptation to stress stimulation in terms of clinical treatment, which can protect against pathological biology and other stresses in diabetes. Overall, the literature suggests that exposure to hypoxic conditions during exercise (simulated altitude of ca. 3000 m) is highly likely to improve the health condition of patients with diabetes. However, there is insufficient evidence for the safety of exposure to short-term hypoxic conditions during exercise in T2DM patients, and further research is needed to develop suitable interventions. Thus, exposure to hypoxic conditions during exercise should be performed with consideration of safety precautions, and patients should be advised by a medical doctor before undertaking exposure to hypoxic conditions.

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REVIEW

Diagnosis, treatment and prevention of type 2 diabetes mellitus in children and adolescents

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Abstract

During the last two decades, there have been several reports of an increasing incidence of type 2 diabetes mellitus (T2DM) in children and adolescents, especially among those belonging to minority ethnic groups. This trend, which parallels the increases in prevalence and degree of pediatric obesity, has caused great concern, even though T2DM remains a relatively rare disease in children. Youth T2DM differs not only from type 1 diabetes in children, from which it is sometimes difficult to differentiate, but also from T2DM in adults, since it appears to be an aggressive disease with rapidly progressive β-cell decline, high treatment failure rate, and accelerated development of complications. Despite the recent research, many aspects of youth T2DM still remain unknown, regarding both its pathophysiology and risk factor contribution, and its optimal management and prevention. Current management approaches include lifestyle changes, such as improved diet and increased physical activity, together with pharmacological interventions, including metformin, insulin, and the recently approved glucagon-like peptide-1 analog liraglutide. What is more important for everyone to realize though, from patients, families and physicians to schools, health services and policy-makers alike, is that T2DM is a largely preventable disease that will be addressed effectively only if its major contributor (*i.e.*, pediatric obesity) is confronted and prevented at every possible stage of life, from conception until adulthood. Therefore, relevant comprehensive, coordinated, and innovative strategies are urgently needed.

Key Words: Type 2 diabetes; Children; Adolescents; Diagnosis; Treatment; Prevention

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Core Tip: Type 2 diabetes mellitus (T2DM) incidence has increased among children and adolescents during the last two decades, especially for minority groups. Youth T2DM is an aggressive disease, associated with high treatment failure rate and early complications. It can be differentiated from type 1 diabetes in obese youth presenting with hyperglycemia, by using both clinical and laboratory clues. T2DM management is based upon the combined application of lifestyle interventions and pharmacological treatments. Nevertheless, prevention seems to be the only way to effectively deal with this disease and this requires preventing pediatric obesity starting as early as before birth and extending throughout childhood.

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INTRODUCTION

Up to 30 years ago, type 2 diabetes mellitus (T2DM) rarely occurred in the pediatric population and was accordingly referred to as "adult-onset diabetes". Gradually, and especially since the turn of the century, several countries started to report an increasing incidence of T2DM in youth, following an increase in both prevalence and degree of pediatric obesity[1-3]. Currently, T2DM is a complex and costly condition in adults, since almost half a billion people worldwide live with the disease, accounting for 90% of diabetes cases. T2DM in these patients can cause renal insufficiency, blindness, lower limb amputation, cardiovascular disease and other complications, causing substantially higher morbidity and mortality than found in the general population.

T2DM in children and adolescents is even more worrying and complex, since it has been proven to be a particularly aggressive form of disease associated with high therapeutic failure rates and leading to much earlier complications than the "adult-onset" form of the disease[4]. Management of these patients in a timely and efficient manner is essential to prevent or at least delay complications and to improve long-term outcomes. Taking into consideration that worldwide prevalence of pediatric overweight and obesity rates have plateaued at high levels in many countries and continue to rise in others[5] and that there is a time lag between obesity onset and T2DM appearance, a further increase in pediatric T2DM is expected in the coming decades. Consequently, not only management but also prevention of obesity and T2DM in children and adolescents should be a top priority for health services and society alike. Otherwise, the next generation might be the first with children of shorter life expectancy than their parents[6].

The present review focuses on the latest available data on diagnosis, treatment and prevention of T2DM in youth and suggests potential areas for future research.

EPIDEMIOLOGY

A rise in pediatric T2DM prevalence is observed worldwide in parallel with the increasing prevalence of obesity in children[7]. In the United States, for instance, T2DM diagnoses accounted for only 3% of all the diabetes diagnoses in children and adolescents in the early 1990's[8]. In the mid-2000's, this percentage rose to 20%[9] and then increased even further, to almost 30%, in the early 2010's, while at the same time type 1 diabetes mellitus (T1DM) incidence also increased[10]. This percentage is even higher (> 50%) for specific ethnic minorities[10]. Similar trends were also observed in other countries[11-13]. Particularly concerning is the increasing incidences reported in China and India, given the fact that these two countries represent one-third of the world's population[14,15].

Despite this increase, T2DM in children and adolescents remains a rare disease. According to data from the SEARCH for Diabetes in Youth Study, its incidence rate in

the United States was 12.5 cases *per* 100000 in 2011-2012[10]. In the United Kingdom and other European countries, its incidence has been much lower, at < 1 case *per* 100000[16], leading some researchers to question the claims of an "epidemic" of T2DM among youth[17]. In addition, a mismatch between the sharp increase in the incidence of childhood obesity and the less severe increase of T2DM could dispute the causal link between the two conditions. This discrepancy can possibly be explained by the long latency period between the onset of obesity and T2DM appearance, combined with the sharp increase of T2DM in early adulthood[18]. In any case, it is generally accepted that pediatric T2DM is emerging as a serious clinical and public health issue[19].

PATHOPHYSIOLOGY AND RISK FACTORS

T2DM is a heterogeneous metabolic disorder characterized by hyperglycemia, insulin resistance, and relatively impaired insulin secretion. Studies in adults have shown that T2DM is caused by complex interactions between social, behavioral and environmental risk factors that affect genetically-susceptible individuals.

Regarding the role of genes in T2DM development, many pieces of the puzzle are still missing but multiple genes seem to play a role (polygenic disease), leading to a strong genetic predisposition. Indeed, a significantly increased risk of T2DM has been demonstrated in close relatives of an affected individual. Monozygotic twin studies showed that if one twin is affected, the other has a 90% chance of developing diabetes[20]. Further, epidemiological studies have shown that more than one-half of youth with T2DM have at least one affected parent[21]. Similarly, the offspring of a parent with T2DM has an almost 3.5-fold increased risk of developing diabetes compared to the risk of an individual without T2DM parental history, and this risk is 6-fold higher if both parents are affected[22].

The role of genetic predisposition in the pathogenesis of T2DM is also illustrated by the differences in the prevalence of the disease in various racial groups. In the United States, for instance, T2DM is much more common in Native, African and Asian Americans as well as in Pacific Islanders and Hispanic children than in the rest of the pediatric population[10]. Similarly, worldwide incidence and prevalence of pediatric T2DM vary substantially among different countries[23]. Sex is another genetically defined "risk-factor" for T2DM, with adolescent girls being 1.3 to 1.7 times more likely than boys to develop the disease for reasons that are not clear[24]. Further, puberty appears to play a central role in T2DM development, due to the physiologically increased insulin resistance of adolescence. It is, thus, no wonder that 40% of T2DM cases are diagnosed in youth between 10 and 14 years of age and the remaining 60% between 15 and 19 years of age[8].

Nevertheless, the rising prevalence of pediatric T2DM observed in recent years cannot be attributed to genetic changes but rather to various environmental factors linked to its pathogenesis[25]. As analyzed in detail in the "Prevention" section below, evidence from several studies suggests that maternal obesity and gestational diabetes mellitus (GDM) increase the risk of obesity and T2DM in the offspring[26]. These, together with other risk factors that abound in the obesogenic environment of modern societies, increase the risk of obesity and visceral fat accumulation in children. It has been demonstrated that increased visceral fat leads to selective insulin resistance through several mechanisms[27]. Initially, euglycemia is preserved through increased insulin production by the pancreatic β-cells. Gradually, in genetically-susceptible individuals with sedentary lifestyle and worsening obesity, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) appear as prediabetic conditions. The resultant hyperglycemia and increased free fatty acid production are toxic for β-cell function (gluco- and lipotoxicity) and progressively, parallel to the worsening insulin resistance, impaired insulin secretion is established[28]. This sequence of events culminates in overt T2DM, with the patient having lost most of his/her pancreatic β-cell function by the time of diagnosis[29]. For reasons largely unknown, the time of progression from insulin resistance to full-blown T2DM is much shorter in adolescents compared to adults, as suggested, for example, by the results of the Treatment Options for T2DM in Adolescents and Youth (TODAY) study[30].

PREDIABETES

Several attempts have been made to identify criteria that will detect patients with prediabetes, *i.e.*, patients that are one step before developing T2DM. The following

criteria have been mostly validated in adult populations but are being used in children and adolescents as well: (1) IFG: Fasting plasma glucose (FPG): ≥ 5.6-6.9 mmol/L; (2) IGT: Plasma glucose: ≥ 7.8-11.0 mmol/L 2 h after a glucose load of 1.75 g/kg (maximum dose: 75 g) in an oral glucose tolerance test (OGTT)[31]; and (3) Hemoglobin A1c (A1C) values between 39 and 47 mmol/mol.

Almost 1 in 5 adolescents in the United States have been found to fulfill one or more of the above criteria, and this percentage was higher in obese subjects of male sex and of minority groups[32]. However, there was little overlap among the subgroups that were diagnosed using abnormal A1C, FPG, or glucose tolerance, thereby throwing the reliability of these criteria for estimating the prevalence of prediabetes in youth into question.

The picture of prediabetes in adolescence is further complicated by the fact that puberty is normally associated with a marked increase in insulin resistance that subsides once adolescence is over. Indeed, in a multi-ethnic prospective cohort of 526 obese youths with IGT, only 8% progressed to T2DM within 3 years, while 65% reverted to normal glucose tolerance[33]. Other factors that might influence progression from a prediabetic state to T2DM are ethnic background and weight changes. For example, in the above study, non-Hispanic black adolescents had a much higher risk of progressing to T2DM compared with white adolescents[33]. Similar studies from Europe have shown an even higher percentage of adolescents converting back to normal glucose tolerance at the end of puberty[34]. Further, expert opinion varies regarding prediabetes management in youth, but lifestyle interventions similar to the ones recommended for patients with established T2DM seem to prevent or delay the development of T2DM[35].

Despite the above limitations, prediabetes conception and especially IFG and IGT are widely used in pediatric populations not only to clarify the natural history of progression to overt T2DM but also as a screening tool for T2DM primary prevention, as described in the "Prevention" section below[35].

CLINICAL PRESENTATION OF T2DM

T2DM in children and adolescents can present clinically in several ways. About one-third of the patients will be identified while not having any of the typical diabetes signs or symptoms. These patients are usually obese individuals in their mid-adolescence who were screened because of one or more positive risk factors or because of glycosuria detected on a random urine test. In addition, they usually have one or more of the typical metabolic syndrome (MetS) characteristics, such as hypertension and dyslipidemia[36].

About one-half of adolescents with T2DM will present with the typical symptoms of hyperglycemia, such as polyuria, polydipsia, and nocturia, just like patients with T1DM. Recent weight loss might also be present but is usually less severe than that observed in T1DM patients[37]. In addition, frequent fungal skin infections, or severe vulvovaginitis due to Candida in adolescent girls can be the presenting complaint[38].

Less than 1 in 10 adolescents diagnosed with T2DM present with diabetic ketoacidosis, namely hyperglycemia, ketonuria, and acidosis[39]. These patients are usually of ethnic minority groups, report polyuria, polydipsia, fatigue, and lethargy, and require admission, rehydration, and insulin replacement therapy. Patients with symptoms such as vomiting can deteriorate rapidly and need urgent evaluation and management. We should keep in mind that a percentage of obese adolescents presenting with diabetic ketoacidosis and diagnosed with T2DM at presentation have T1DM rather than T2DM and will need lifelong insulin treatment[40].

Hyperosmolar hyperglycemic state (also known as hyperosmolar hyperglycemic nonketotic syndrome) is fortunately the rarest presenting clinical picture of children with T2DM. It is characterized by severe hyperglycemia (plasma glucose > 33 mmol/L), increased serum osmolality (> 330 mOsm/kg) and severe dehydration, with little or no ketonuria. It is a medical emergency, with high morbidity and mortality if not adequately treated[41].

DIAGNOSIS

Since T2DM represents one of several different diabetes types, diagnosing a child or adolescent with the disease is a two-step process. Firstly, one has to confirm the diagnosis of diabetes and secondly, to establish that it is diabetes type 2.

According to the American Diabetes Association (ADA), the criteria used to diagnose diabetes in youth are the same as those used in adult populations[42]. There are four possible ways to diagnose diabetes and each, in the absence of hyperglycemia symptoms, must be confirmed on a different day by any one of the other three: (1) FPG ≥ 7.0 mmol/L; (2) 2 h post-OGTT plasma glucose ≥ 11.1 mmol/L. It should be noted that OGTT has poor reproducibility in adolescents, with a concordance rate of < 30% between tests performed a few weeks apart[43]; (3) random plasma glucose ≥ 11.1 mmol/L in the presence of diabetes symptoms. If such symptoms are not present, hyperglycemia diagnosed incidentally or under stress conditions (*e.g.*, acute infection or surgery) may be transitory and should not be regarded as diagnostic of diabetes. In such cases, repeat exam on a subsequent day will help diagnostically; and/or (4) A1C ≥ 48 mmol/mol. This criterion remains controversial since, in some but not all, studies it identifies a population that does not overlap entirely with that identified by FPG or OGTT[44-46]. In addition, A1C must be measured by using a laboratory-based National Glycohemoglobin Standardization Program-certified methodology and not a point-of-care device, in order to be reliable.

It is important to remember that none of the above criteria has ever been validated in pediatric population studies but they have been extrapolated from adult definitions. Such studies in youth would take a substantial period of time, and may prove unfeasible to perform[45].

Once the diagnosis of diabetes is established, the next important step is to differentiate T2DM from T1DM as well as from other more rare diabetes types. This distinction is not merely of academic importance but of clinical importance as well, since different types of diabetes require a different management approach, at least in the long-term[47]. Since there is considerable overlap between T2DM and T1DM, a combination of history clues, clinical characteristics and laboratory studies must be used in order to reliably make the distinction, which is not always possible at the beginning (Table 1). Such clues include: (1) Age. T2DM patients usually present after the onset of puberty, at a mean age of 13.5 years. By contrast, almost one-half of T1DM patients present before 10 years of age[9]; (2) Family history. A reported 75%-90% of patients with T2DM have an affected first- or second-degree relative[31], while the corresponding percentage for patients with T1DM is less than 10%; (3) Ethnic group. Youth belonging to minority groups such as Native American, African American, Hispanic, and Pacific Islander run a much higher risk of developing T2DM compared to Caucasians[24]; (4) Body weight. Adolescents with T2DM are usually obese [body mass index (BMI) ≥ 95 percentile for age and sex]. In contrast, children with T1DM are usually of normal weight and may report a recent history of weight loss; although, up to 25% are overweight or obese[7]; and (5) Clinical findings. Patients with T2DM usually present with features of insulin resistance and MetS, such as acanthosis nigricans, hypertension, dyslipidemia, and polycystic ovary syndrome (PCOS)[48]. Such findings are rarely encountered in youth diagnosed with T1DM. For instance, a study in the United States showed that up to 90% of youth diagnosed with T2DM had acanthosis nigricans, in contrast to only 12% of those diagnosed with T1DM[49].

In addition to the above history and clinical clues, laboratory tests that can help include those for: (1) Pancreatic autoantibodies. Since T2DM is not immunologically mediated, the identification of one or more pancreatic (islet) cell antibodies in a diabetic obese adolescent supports the diagnosis of autoimmune diabetes[50]. Antibodies that are usually measured include islet cell antibodies (against cytoplasmic proteins in the β-cell), anti-glutamic acid decarboxylase, and tyrosine phosphatase insulinoma-associated antigen 2, as well as anti-insulin antibodies, provided that insulin replacement therapy has not been used for more than 2 wk. In addition, a recently described β-cell-specific autoantibody to zinc transporter 8 is commonly detected in children with T1DM and can aid in their differential diagnosis[51,52]. One should keep in mind, though, that up to one-third of T2DM children can have at least one detectable β-cell autoantibody and, thus, complete absence of diabetes autoimmune markers is not a prerequisite for the diagnosis of T2DM in children and adolescents[53,54]; (2) Ketoacidosis. Since patients with T1DM are more prone to develop ketoacidosis at the time of diagnosis, measurement of venous pH and urinary ketones could help differentiate between T2DM and T1DM, especially in the presence of typical symptoms (*e.g.*, polydipsia, polyuria, and signs of dehydration). Of course, it should be remembered that up to 10% of adolescents with T2DM can have a similar initial clinical presentation[55]; and (3) Insulin and C-peptide levels. A low C-peptide level (< 0.2 nmol/L) detected in newly diagnosed diabetic youth strongly suggests T1DM[56]. Insulin levels can also be used, provided that insulin therapy has not been initiated.

The challenges of differentiating between T2DM and T1DM were demonstrated in a

Table 1 Clinical and laboratory findings of type 1 and type 2 diabetes mellitus and maturity-onset diabetes of the young in children and adolescents

Parameter	T1DM	T2DM	MODY
Prevalence	Common, increasing	Rare, increasing	Rare, stable
Ethnicity	Mainly Caucasian	Mainly minority groups	All
Inheritance	Multigenic	Multigenic	Autosomal dominant
Family history	5%-10% positive for T1DM	75%-90% positive for T2DM	100% positive for MODY
Sex	Male = Female	Male < Female	Male = Female
Age at presentation	Childhood-adolescence	Adolescence	Before 25 yr of age
Body habitus	Usually normal weight	Mostly obese	Various
Acanthosis nigricans	Rare	Very common	Absent
Onset	Usually acute, severe	Usually insidious, rarely acute	Insidious
Ketosis at onset	Common	5%-10%	Rare
Insulin, C-peptide	Decreased or absent	Variable	Detectable
Insulin sensitivity	Normal	Decreased	Normal
HLA-DR3/4 association	Strong	None	None
Pancreatic autoantibodies	85%-100%	< 10%	Rare
Insulin dependence	Permanent	Variable	Rare
Associated disorders	Autoimmune disorders (*e.g.*, Hashimoto, vitiligo, celiac disease)	MetS components (*e.g.*, lipid disorders, hypertension, PCOS, sleep apnea, *etc.*)	Depending on type, may present with exocrine pancreas insufficiency, urogenital malformation, *etc.*

HLA: Human leukocyte antigen; MetS: Metabolic syndrome; MODY: Maturity-onset diabetes of the young; PCOS: polycystic ovary syndrome, T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus.

multicenter study of 2291 subjects aged < 20 years with recently diagnosed diabetes that were classified based upon presence or absence of β-cell autoimmunity and insulin sensitivity[49]. More than 70% of the patients fell into the traditional autoimmune plus insulin-sensitive T1DM (55%) or non-autoimmune plus insulin-resistant T2DM categories (16%). Almost 20% of the subjects were diagnosed with autoimmunity and insulin resistance, and were considered to be obese individuals with T1DM. The smallest group (10%) comprised insulin-sensitive patients in the absence of pancreatic autoimmunity that could either be patients with type 1B or with monogenic diabetes, thus requiring further testing.

Maturity-onset diabetes of the young (MODY) is the most common form of monogenic diabetes. MODY is a clinically heterogeneous group of disorders characterized by non-insulin dependent diabetes together with lack of pancreatic autoimmunity. It is a congenital disorder with autosomal dominant transmission that is usually diagnosed in childhood or early adulthood. MODY accounts for at least 1.2% of all cases of diabetes in the United States in people aged 20 years and younger and 2.5% in the United Kingdom[57,58]. Eleven different MODY types have been described thus far, caused by mutations in several genes and with the most common types being MODY3 (*HNF-1a* gene mutations) and MODY2 (glucokinase gene mutations)[59]. MODY should be suspected in young (< 25 years) patients presenting with non-insulin-dependent diabetes (detectable C-peptide), usually without the typical comorbidities of obesity and MetS, without pancreatic autoantibodies, but with a strong family history of diabetes for more than one generation (Table 1). Its diagnosis requires genetic testing by direct sequencing of the suspected gene[60].

MANAGEMENT

Even if studies regarding long-term outcome of adolescent patients with T2DM are scarce, they show that youth T2DM is a particularly aggressive form of disease associated with early emergence of complications, not only in absolute terms[30,61,62] but also compared to youth with T1DM[63] or to adults with T2DM[4]. In addition, adolescents with T2DM exhibit a faster rate of deterioration of β-cell function[64] and greater extent of insulin resistance than adults with similar adiposity, presenting a decreased response to insulin sensitizers and a high therapeutic failure rate[65,66]. It is, therefore, essential to manage these patients timely and efficiently, in order to avoid or delay complications and to improve long-term outcomes.

Only a few studies have evaluated the management of T2DM in the pediatric age group. The largest among them is the TODAY study, which showed that oral agent monotherapy failed to maintain glycemic control in almost half of the patients evaluated, within a year of the treatment initiation[67]. According to similar evidence from other studies, it seems that the best approach to manage adolescents with T2DM is a combination of non-pharmacologic and pharmacologic interventions, with close monitoring and follow-up[68]. This approach is consistent with the Consensus Guidelines published in 2013 by the American Academy of Pediatrics, Pediatric Endocrine Society, Academy of Nutrition and Dietetics, and American Academy of Family Physicians[69], updated in 2018 by the International Society for Pediatric and Adolescent Diabetes (ISPAD)[19], and in 2020 by the ADA[70].

The best health services for an adolescent with T2DM can be provided by a multidisciplinary team consisting of an endocrinologist, a nurse educator, a dietitian, a mental health professional, and an exercise physiologist. In countries with limited resources, primary care physicians can manage these patients based on published guidelines[69]. Under these circumstances, at least, patients with poor glycemic control should be referred to an endocrinologist and a diabetes educator. It must be emphasized that most of the non-pharmacological interventions that are needed to manage T2DM in youth require active involvement of the entire family, if they are to be successful. In addition, all youth with T2DM and their families must receive comprehensive diabetes self-management education and support.

Management goals

The goals of managing an adolescent with T2DM are the following: (1) To achieve and maintain near-normal glycemic levels with minimal hypoglycemic episodes; (2) To improve body weight, insulin sensitivity and possibly insulin secretion, in order to achieve better glycemic control and improved overall health; (3) To identify and manage the disease in a timely manner and, if necessary, comorbidities and complications such as hypertension, dyslipidemia, hepatic steatosis, nephropathy, and retinopathy; and (4) To prevent or delay, as much as possible, macrovascular complications of T2DM, such as cardiovascular disease and stroke.

These goals can be achieved through the successful implementation of non-pharmacologic and pharmacologic measures. In extreme cases, surgical intervention should be considered.

NON-PHARMACOLOGIC INTERVENTIONS

It is well established that increased fat mass and especially visceral fat is responsible for many of the features that characterize children with MetS and overt T2DM[71]. In adults with T2DM, weight loss has been shown to reduce peripheral insulin resistance and to increase insulin secretion by the β-cells[72]. Similarly, in obese children without T2DM, a BMI decrease of ≥ 0.5 kg/m^2 was shown to improve insulin sensitivity, while the opposite was true for BMI increase[73]. In a study with adolescent patients with T2DM that were treated for 1-4 mo with very low-calorie diets, both BMI and A1C dropped and pharmacologic agents were discontinued in all but 1 of the 20 patients[74]. Obviously, such restrictive diets are very difficult to implement for longer periods of time and can lead to significant nutrient deficiencies in children and adolescents.

The goal for children and adolescents with T2DM should be a 7%-10% reduction in BMI for those that have completed linear growth, or a BMI < 85th percentile for age and sex for those that are still growing[68]. For the latter, weight maintenance could be enough, since it would lead to BMI reduction as the child grows taller. However, since most youth diagnosed with T2DM are in their mid-adolescence and present with severe obesity, weight reduction rather than maintenance should be the long-term

goal. A sensible approach is to start with weight maintenance for some months as the first step and continue with weight loss at a rate of 0.5-1 kg *per* month. For older adolescents that have completed puberty, the recommended weight loss rate is the same as for adults, *i.e.* 0.5-1 kg *per* week. One should keep in mind that this goal can prove quite challenging for many obese adolescents and their families, and changes in both diet and physical activity have to be implemented.

Diet

In order to avoid macro- or micronutrient deficiencies, specific dietary intervention programs should be carried out by an experienced nutritionist/dietitian with knowledge and experience in nutritional management of youth with diabetes. Consultation with a dietitian is particularly important for patients who fail to achieve adequate glycemic control and require treatment intensification. The whole family must be encouraged to make gradual dietary changes consistent with healthy eating recommendations, and healthy parenting practices related to diet and activity should be applauded. Dietary recommendations must be adjusted to each family's possible cultural or financial constraints and should focus on the following[75,76]: (1) elimination of sugar-sweetened soft drinks and fruit juices; (2) reduced consumption of processed and prepackaged foods; (3) decreased intake of refined, simple sugars and corn syrup; (4) reduced saturated and total fat intake; (5) increased fruit and vegetable intake; (6) increased consumption of fiber-rich foods, such as whole grain products and legumes; (7) preferable consumption of foods with low glycemic index; (8) better portion control; and (9) elimination of meals eaten away from home or while screen watching.

It is also important to remember that patients are more likely to follow a diet that is adapted to their preferences and habits and, therefore, dietary interventions must be individualized and have goals that are both measurable and achievable. In order to assess progress and to keep both the patient and the family motivated, frequent visits to the dietitian (*e.g.*, every 4 wk) are recommended[77].

Physical activity

Increased physical activity has a significant role in the management of youth with T2DM, since it not only helps in weight reduction but also increases insulin sensitivity and improves blood glucose control[78,79]. Youth with T2DM should be instructed to gradually increase their physical activity towards a goal of 1 h daily. Exercise must include moderate-to-vigorous aerobic activities and, in addition, strength training at least three times a week. In addition, the patient should engage in daily efforts to be more active physically, such as walking to school instead of taking the school bus, using stairs instead of elevators, doing house and yard work, and so on. At the same time, nonacademic screen time (*e.g.*, television, video games, social media) must be decreased to less than 2 h a day and other sedentary behaviors should be kept to a minimum[70]. Just like dietary changes, physical activity interventions have to be individualized for each patient and family, and should be enjoyable and achievable.

PHARMACOLOGICAL AGENTS

For several years, the only agents approved by the United States' Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of T2DM in children and adolescents were metformin and insulin. Metformin is a biguanide that increases insulin-mediated glucose uptake in the peripheral tissues and decreases hepatic glucose production, thereby promoting decrease of plasma glucose levels[80]. In addition, it has been shown to help in modest weight loss, albeit with only a temporary effect[81]. On the other hand, insulin therapy is used in the initial management of T2DM patients who present with severe hyperglycemia and ketosis or ketoacidosis or in patients that have mixed features of T1DM and T2DM, as described below.

Both the FDA and EMA, based on the promising results of the Evaluation of Liraglutide in Pediatrics with Diabetes clinical trial[82], approved the use of liraglutide for the treatment of T2DM in youth in 2019. Liraglutide is a glucagon-like peptide-1 (GLP-1) analog that acts by increasing glucose-dependent insulin secretion from pancreatic β-cells following ingestion of a meal, thereby ensuring an appropriate insulin response and avoidance of hyperglycemia. It may also promote modest weight loss, due to delayed gastric emptying and central effects on appetite[82]. A problem of the GLP-1 analogs treatment is the need for daily subcutaneous injection, which may be solved in the near future by long-acting analogs requiring only once-weekly

administration or oral preparations for adolescents.

Many other anti-hyperglycemic agents have been approved for use in adults but none in youth with T2DM, except for sulfonylureas (*e.g.*, glimepiride) in some countries[83]. Therefore, such drugs should not be used in adolescents outside research trials until more data regarding their safety and efficacy are available. For instance, the TODAY clinical trial showed that administration of rosiglitazone (a thiazolidinedione) with metformin failed to improve the lipid profile and the cardiovascular risk in youth with T2DM[30]. Clinical trials with adolescents are underway, testing the safety and efficacy of agents belonging to various drug categories. A detailed description of all the anti-hyperglycemic agents used in adult patients with T2DM is beyond the scope of this review.

Initial treatment

Some obese adolescents with T2DM will present with diabetic ketoacidosis and several others will present with features of both T1DM and T2DM. For this reason, immediate therapy should address hyperglycemia and possible metabolic derangements, irrespective of ultimate diagnosis[68]. According to the Consensus Guidelines published by the ADA and ISPAD, initial treatment of youth with T2DM should include metformin and insulin, either alone or in combination[19,68,70]. The decision on the initial treatment is individualized, as follows (Figure 1): (1) If the patient has no symptoms and A1C is < 69.4 mmol/mol, metformin is the treatment of choice, accompanied by lifestyle modifications. The initial dose is 500 mg once a day (taken with meals) and can be gradually increased by 500 mg every week, depending on patient tolerability, up to the maximal dose of 1000 mg BID or 850 mg TID (or 2000 mg once a day of extended-release metformin, if available). This slow titration can reduce gastrointestinal side effects; (2) If the patient has marked hyperglycemia (A1C ≥ 69.4 mmol/mol and/or blood glucose ≥ 13.9 mmol/L) together with related symptoms (polyuria, polydipsia, nocturia, weight loss) but without ketoacidosis, combination therapy with basal insulin and metformin is suggested; and (3) Patients who present with ketosis or ketoacidosis should initially be treated with insulin alone without metformin. A variety of insulin regimens is being used but once-a-day intermediate or basal insulin (0.25-0.5 U/kg starting dose) is often effective and well tolerated by the patient. Metformin should be added only after ketoacidosis has subsided and glucose levels have reached near-normal with insulin therapy. Since many youth with T2DM can be successfully weaned off insulin and treated with metformin alone[84], a gradual transition can usually be achieved over 2-6 wk by decreasing the insulin dose each time metformin is increased, simultaneously ensuring that glycemic targets are met.

The goal of treatment is to achieve an A1C of < 53 mmol/mol in most adolescents with T2DM and < 47.5 mmol/mol in others, for example those with shorter diabetes duration and less severe obesity[19,68,70]. Just like lifestyle modifications, treatment goals can initially be individualized. If, for example, the above targets seem unrealistic for a specific individual or if there is increased hypoglycemia risk, one can start with a higher target (*e.g.*, A1C < 64 mmol/mol) and then gradually decrease it[69]. In addition to A1C levels, FPG levels can be used as an indication of adequate therapy, with FPG < 7.2 mmol/L as a general goal.

A1C is typically measured every 3 mo. Self-monitoring of blood glucose can be done at home with a glucometer, at least three times *per* day in children who are on insulin therapy, whose dosage or treatment regimen is changing, or who are not meeting goals for glycemic control[69]. Use of continuous glucose monitoring could be considered in patients failing to attain glycemic targets and in those on a multiple daily injection regimen, although our knowledge on its use in adolescents with T2DM is just starting to evolve[85].

Intensification of therapy

Patients who fail to achieve adequate glycemic control require re-evaluation of their management. The initial steps include more intense efforts for lifestyle modifications, review of medication adherence, and dealing with possible barriers, as well as more frequent blood glucose measurements. If all these measures fail, a change in medication is the next step (Figure 1).

For patients who fail to achieve glycemic control 3-6 mo after intensification of lifestyle measures and monotherapy with metformin at the maximal tolerated dose (up to 1000 mg BID), basal insulin can be added, with liraglutide as an acceptable alternative. The advantage of insulin is the much greater clinical experience we have with its use in diabetic children and adolescents but, at the same time, one has to consider that it can cause weight gain and hypoglycemia, and requires frequent dose adjustment. Liraglutide, on the other hand, is a rather new anti-diabetic agent for teens

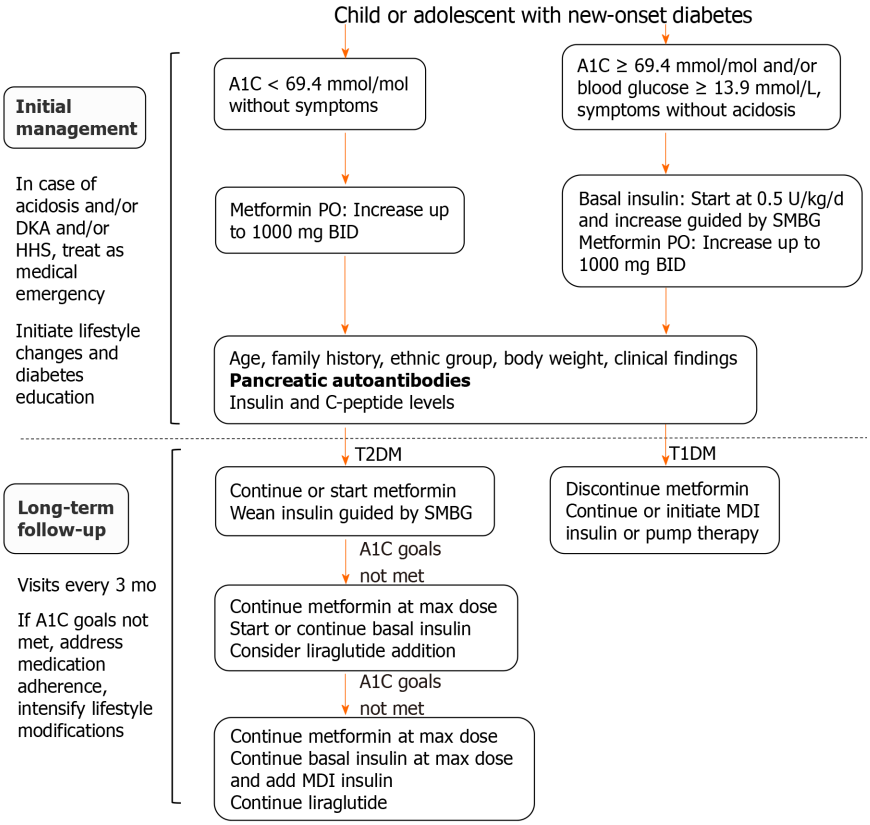

Figure 1 Management of new-onset diabetes in obese youth. A1C: Hemoglobin A1c; BID: Twice *per* day; DKA: Diabetic ketoacidosis; HHS: Hyperosmolar hyperglycemic state; IV: Intravenous; MDI: Multiple dose injection; PO: Per os; SC: Subcutaneous; SMBG: Self-monitored blood glucose; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus.

but there are no long-term data on its use and it might be more costly than insulin. It seems, though, to be at least as effective as basal insulin and may help obese diabetic youth to lose weight; it also has an easy dosing scheme[86].

For adolescents who fail to achieve adequate glycemic control despite intense lifestyle interventions and combination therapy of metformin (at maximal tolerated dose) with basal insulin (at a dose ≤ 1.5 U/kg/d), either liraglutide or prandial rapid-acting insulin can be added. Both approaches are acceptable, but liraglutide (at a starting dose of 0.6 mg/d) has the advantage of a single daily injection compared to the three injections required for prandial insulin. This is a rather important point to remember, considering the poor adherence of diabetic youth to insulin therapy[87].

It should be noted that adolescents with T2DM might have severe insulin resistance and, thus, basal insulin doses above 1.5 U/kg/d may be needed to achieve adequate glycemic control, particularly for those with higher A1C and glucotoxicity and those in mid- to late puberty[68]. If intense lifestyle interventions together with combination therapy of metformin, insulin, and liraglutide at maximal doses fail, bariatric surgery is a reasonable option.

SURGICAL THERAPY

Weight loss surgery is a rather new therapeutic approach for severely obese adolescents (BMI ≥ 120% of the 95th percentile for age and sex) with T2DM and/or other serious comorbidities and who fail to achieve glycemic control despite intensive lifestyle and pharmacologic intervention[68,75]. Several different techniques have been employed, such as gastric bypass, sleeve gastrectomy and adjustable banding, with good safety and efficacy results, if performed in experienced centers. T2DM usually subsides after surgery and remains in remission for some years, but relevant long-term data are lacking. As an example, in a multicenter, prospective study of bariatric surgery in severely obese adolescents with T2DM, diabetes resolved after surgery in 95% and remained in remission in 90% of a subgroup of them at 5 years later[88]. In the recent Teen-Longitudinal Assessment of Bariatric Surgery/TODAY study comparison,

adolescents treated with bariatric surgery demonstrated better glycemic control compared to age-, sex- and BMI-matched patients managed with medical therapy alone, but 30% of them required readmission and/or reoperation[89]. It is obvious that long-term follow-up and further research are needed regarding eligibility criteria, possible short- and long-term benefits and risks, as well as the optimal timing of bariatric surgery for obese youth with T2DM; thus far, the data are promising.

SCREENING FOR COMORBIDITIES AND COMPLICATIONS

When diagnosed with T2DM, many adolescents have already developed one or more of the MetS components, such as hypertension, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), obstructive sleep apnea, and PCOS[1]. These comorbidities, together with hyperglycemia and increased insulin resistance, rapidly aggravate the adolescent's health status and accelerate the appearance of microvascular complications such as nephropathy, retinopathy, and neuropathy, as well as of macrovascular complications and cardiovascular disease. Therefore, screening for and management of comorbidities and complications both at the time of diagnosis and in the course of the disease are essential components of T2DM management in youth (Table 2)[70,90].

Screening for hypertension should start as early as possible after T2DM diagnosis, since 15%-30% of obese adolescents with T2DM are diagnosed with high blood pressure (BP), with some being hypertensive already at diagnosis[91]. BP should be measured with an appropriately sized cuff at all routine health visits. If the BP remains elevated despite lifestyle interventions, anti-hypertensive therapy must be initiated, such as by prescription of an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin II receptor blocker[68,70]. Regarding dyslipidemia, all pediatric patients with T2DM should undergo lipid profile assessment at the time of diagnosis (after hyperglycemia has been controlled) and every year thereafter. Non-pharmacologic interventions can improve lipid levels to some extent. If these measures fail and low-density lipoprotein cholesterol remains elevated [≥ 130 mg/dL (≥ 3.36 mmol/L)], pharmacologic treatment (*e.g.*, a statin) can be started[70]. Youth with T2DM should also be screened annually for NAFLD, along with measurement of aminotransferase levels and possibly liver ultrasonography. Weight reduction is the only established treatment for this comorbidity[70].

Microvascular disease is the hallmark of hyperglycemia in both adults and children with T2DM and the relative risk increases with worse glycemic control and disease duration. In a children population-based cohort study, major complications related to microvascular dysfunction (*i.e.*, dialysis, blindness, or limb amputation) started to manifest 10 years after the T2DM diagnosis[92]. Regarding nephropathy, youth with T2DM should be screened annually for albuminuria by measuring the urine albumin-to-creatinine ratio in a random urine sample. Treatment with an ACE inhibitor is recommended for patients with increased albuminuria and elevated BP[70]. Diabetic retinopathy must be excluded by annual screening of youth *via* dilated eye examination and/or retinal imaging. Annual screening for diabetic neuropathy is also indicated, beginning at diagnosis, and includes a careful neurologic examination of the sensory nerves[70].

Regarding macrovascular complications, several lines of evidence suggest that children and adolescents with T2DM are at high risk of atherosclerosis and premature ageing of their cardiovascular system. Indeed, they have increased carotid intima media thickness, arterial stiffness, and left ventricular wall thickness compared to obese non-diabetic or normal weight controls[93-96]. Further, it was shown that adults with T2DM who were diagnosed between 15 and 30 years of age have double cardiovascular mortality compared to patients of similar age with T1DM of similar duration[63]. In order to reduce macrovascular disease, youth with T2DM should be screened and aggressively treated if found to have hypertension or dyslipidemia. In addition, smoking avoidance or cessation should be strongly encouraged. Better glycemic control should also be a target even though it has not yet been formally linked with improved cardiovascular outcome in T2DM youth. Routine screening of asymptomatic diabetic children and adolescents with electrocardiography, echocardiography, or stress testing is not recommended[70].

Table 2 Routine monitoring of children and adolescents with type 2 diabetes for comorbidities and chronic complications

Evaluation	Test performed	Testing frequency
Hypertension	BP measurement with appropriately-sized cuff	At the time of diagnosis and at each routine visit; more frequently if elevated
Dyslipidemia	Non-fasting or fasting lipid panel	At diagnosis once glycemic control is achieved. Annually thereafter, more frequently if abnormal
NAFLD	Liver transaminases	At diagnosis and annually thereafter
Retinopathy	Dilated eye examination or retinal imaging	At diagnosis and annually thereafter, or as per ophthalmologist's advice
Nephropathy	In a spot specimen urine albumin-to-creatinine ratio	Repeat annually. If abnormal, repeat on at least two occasions during the next 3-6 mo
Neuropathy	Foot examination (pulses and ankle reflex); sensory testing for vibration (tuning fork) and sensation (10-g monofilament)	Repeat annually. If abnormal, refer to neurologist
Psychosocial assessment	Screen for depression, eating disorders, risk-taking behaviors, or other psychosocial dysfunction	Repeat at each routine visit or as needed. If abnormal, refer to mental health professionals

BP: Blood pressure; NAFLD: Non-alcoholic fatty liver disease.

THE IMPORTANCE OF PATIENT'S ADHERENCE

Despite efforts of medical services, evidence shows that only a few patients with T2DM have an acceptable lifestyle modification and medication adherence level in the long-term. For example, data in adult populations have shown that adherence to dietary recommendations is < 65% and even lower (< 30%) to physical activity recommendations[97]. In addition, adherence to treatment with oral hypoglycemic agents and insulin therapy ranges between 36%-93% and between 20%-80%, respectively[97]. Several factors, such as complexity of treatment, have been implicated in low adherence in adults with T2DM[98]. What seems to be very important in both adult and adolescent populations though, is psychological factors such as depression and anxiety[99,100]. Therefore, it is important to support these young patients both emotionally and socially if we are to improve adherence and glycemic control. In addition, family support together with pairing the medication regimen with daily routines have been suggested by adolescents themselves to be important strategies for medication adherence improvement[101].

PREVENTION

According to Hippocrates of Kos (460-377 BC) "preventing is better than treating". In the case of pediatric T2DM with the difficult management and early serious complications, this could not be more true. As detailed above, there are several risk factors that increase the likelihood of early T2DM development. Some of them, such as genetic predisposition and ethnicity, cannot be altered, while others are potentially modifiable and could, therefore, be targets for preventive initiatives (Figure 2).

Mounting evidence indicates that youth T2DM follows the Developmental Origins of Health and Disease concept, according to which various events during critical periods, such as *in utero* or early years of life, predispose the developing organism to health or disease later in life[102]. Therefore, a primordial prevention approach aiming at modifiable risk factors, starting as early as before birth and extending throughout childhood, can have the greatest impact on preventing T2DM.

Intrauterine life

Both cohort- and registry-based studies have shown that maternal overweight and obese status are associated with T2DM in offspring, irrespective of various confounding pre-existing or pregnancy-related conditions[103-106]. In one of the largest such studies, for instance, Lahti-Pulkkinen *et al*[104] found that children born to obese or overweight women had a 3.5- and 1.4-fold higher incidence of T2DM respectively, compared to those born to normal-weight women. Further, several studies have linked high pregravid BMI or increased weight gain early in pregnancy to increased risk of childhood obesity in the offspring[107-109], which predisposes to early T2DM develop-

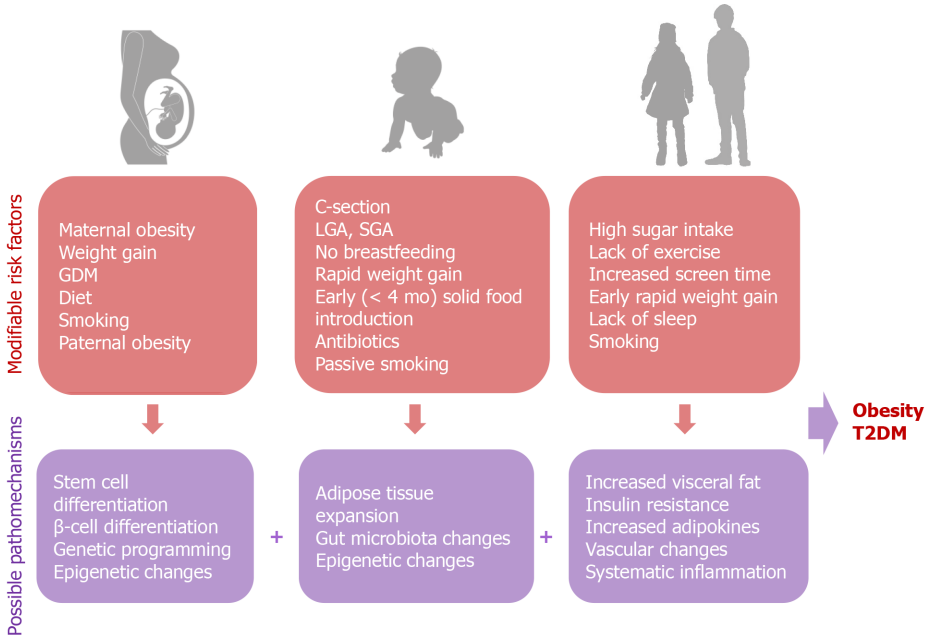

Figure 2 Modifiable risk factors and possible pathomechanisms in different age groups, leading to obesity and pediatric type 2 diabetes mellitus. C-section: Cesarian section; GDM: Gestational diabetes mellitus; LGA: Large-for-gestational age; SGA: Small-for-gestational age; T2DM: Type 2 diabetes mellitus.

ment. These results show that one of the earliest points of youth T2DM prevention is the reduction of overweight and obese status during pregnancy.

Obesity during pregnancy also increases the risk of GDM in the mother. Compared with normal-weight women, obese mothers have more than a 3-fold increased risk of developing GDM[110]. A substantial body of evidence, both in minority groups and in the general population, has shown that offspring of women with GDM are at increased risk of T2DM and obesity, irrespective of the mother's weight status during pregnancy[105,111]. In addition, not only overt GDM but also prediabetic conditions during pregnancy have been linked to glucose abnormalities and insulin resistance in the offspring[112]. Protection of the fetus from a diabetic intrauterine environment is, therefore, of paramount importance to prevent prediabetic conditions and T2DM in childhood and adolescence. Other risk factors such as maternal diet[113], maternal smoking[114], and even paternal obesity[115] should also be considered (Figure 2).

Addressing obesity and diabetes during pregnancy will not only protect youth from T2DM but could also prove beneficial at the population level by mitigating the so-called "vicious cycle of diabetes and obesity", first described by Pettitt and Knowler[116]. According to this concept, obese and diabetic pregnant women confer to their offspring the predisposition for developing obesity and T2DM, and the offspring, in turn, when at reproductive age, will give birth to another generation of obese diabetic individuals, thus perpetuating a transgenerational cycle of disease[117].

Early life

In order to prevent youth T2DM, one should clearly focus on intrauterine life, but postnatal exposures seem to play an equally important role. Diet and environment during the first 1000 d of each individual, from conception to the second birthday, have gained much attention as a window of opportunity for T2DM prevention[118]. For instance, breastfeeding has been shown to have a strong protective effect against early-onset T2DM in various populations[119,120]. In addition, it seems that breastfeeding for ≥ 6 mo mitigates the risk of an *in utero* diabetes exposure, regarding childhood adiposity and fat distribution[121], as well as prediabetes and MetS development[122], suggesting a specific protective effect among high-risk offspring.

Both high and low birth weight[123,124], preterm birth[125], as well as rapid weight gain during the first months of life[126,127] have been linked to increased risk of obesity later in life and to insulin resistance and glucose metabolism disturbances, thus predisposing to T2DM. Several other early life risk factors, such as Cesarean section[128], antibiotic exposure[129], secondhand smoking[130], and early (< 4 mo of age) solid food introduction[131], have been linked to increased obesity risk. Even if a direct link to glucose abnormalities and T2DM development has not been established, preventive

initiatives including these factors could prove helpful in preventing pediatric obesity and thus T2DM (Figure 2).

From their first birthday on, children can and should eat their meals at the family table. Following the diet and physical activity recommendations for children of this age is important to avoid later obesity. Especially important seems to be the preschool period since, as Geserick *et al*[132] showed, rapid weight gain between 2 and 6 years of age is associated with a much higher risk of overweight or obesity status in adolescence, thereby increasing the risk of early T2DM.

Childhood and adolescence

Measures to tackle T2DM in childhood and adolescence are based on obesity prevention, given the etiological connection between increased body fat and MetS and T2DM. Programs to limit childhood obesity are mainly school-based and seem to yield the best results in children younger than 12 years, although robust conclusions cannot be drawn given the heterogeneity of relevant studies. Food choices in this age group can be improved by measures that involve parents and teachers alike, such as healthier school meals, taxes on simple sugars, and restriction of unhealthy food advertisements aimed at children. Nutritional interventions should be combined with programs targeting increased physical activity in order to achieve the best long-term outcome[133,134].

For adolescents, interventions include improving eating habits, increasing physical activity, and restricting sedentary and screen time. In this age group, school-based interventions have proven more effective when the adolescents were addressed directly[135]. Behavior-oriented prevention programs have shown limited long-term effects thus far[136]. Further, interventions to improve the current obesogenic environment could prove essential in the fight against pediatric obesity and T2DM. Several environmental and social factors, such as length of the street the children live on, accessibility to playgrounds and sports facilities, population density and socioeconomic status of the neighborhood, influence children's BMI, even if only to a limited extent[137,138].

Screening for prediabetes

Primordial prevention is important, as described above. Equally important can be primary prevention through screening strategies aiming to identify prediabetes in youth and avert progression to T2DM. To date, no studies in a pediatric age group have examined if early diagnosis improves T2DM long-term outcome. However, there is indirect evidence from adult studies showing that lifestyle interventions can delay or even prevent the onset of T2DM[139].

Since generalized population screening of obese youth is unlikely to be cost-effective in most populations, the ADA and ISPAD recommend screening only high-risk individuals[68,70,140]. These include asymptomatic overweight or obese children and adolescents after the onset of puberty or at ≥ 10 years of age (whichever occurs first) if they have one or more of the following risk factors: (1) family history of T2DM in a first- or second-degree relative; (2) minority race/ethnic group (Native American, African American, Hispanic, Asian American, Pacific Islander); (3) maternal history of diabetes or GDM during the child's gestation; and/or (4) conditions or signs associated with insulin resistance (*i.e.* hypertension, dyslipidemia, acanthosis nigricans, PCOS, small-for-gestational age status at birth).

According to ADA recommendations, this screening should be repeated at least every 2-3 years, or earlier if BMI is increasing, and should be done by measuring A1C and FPG or by performing an OGTT. Abnormal results must be confirmed either by the same test on a different day or by performing a different test.

FUTURE PERSPECTIVES

Pediatric T2DM incidence has increased considerably and is expected to rise even further in the decades to come. According to a study that was based on data from the SEARCH for Diabetes in Youth Study, the number of youth with T2DM in 2050 is likely to increase 4-fold compared to the levels in 2010, with substantially larger numbers among minority youth[141]. This trend, if verified, will lead to a much heavier economic and societal burden, with many young adults having serious health conditions.

In order to avoid such an adversity, more measures have to be taken in the near future regarding both management and prevention of pediatric obesity and T2DM.

Regarding treatment, clinical trials of various anti-hyperglycemic agents used in adults from different categories, such as sodium-dependent glucose cotransporters inhibitors and dipeptidyl peptidase-4 inhibitors, are underway in pediatric populations and results are expected in the coming years. For instance, there are ongoing phase 3 studies of canagliflozin (NCT03170518), dapagliflozin and saxagliptin (NCT03199053) as well as of linagliptin and empagliflozin (NCT03429543) in patients with T2DM of ages between 10 and 18 years. The first results regarding empagliflozin pharmacokinetic characteristics in teens have already been published[142]. Further, studies in pediatric patients are needed with GLP-1 analogs designed for once-weekly dosing that are already available for use in adults as well as oral preparations, in order to help adolescents with poor adherence to liraglutide.

Even more important than improving pediatric T2DM management is optimizing its prevention. In order to develop effective preventive approaches, we need to elucidate mechanisms linking genetic, epigenetic, social, environmental and other risk factors with T2DM pathogenesis. To achieve this and to move from association to causation, better studies have to be designed, such as longitudinal cohorts starting even before birth. Such an example is the EarlyBird cohort which recruited 307 healthy children in the United Kingdom at 5 years of age and followed them throughout childhood, and which very recently showed that pancreatic β-cell defects predate insulin resistance in the onset of prediabetes[143].

In addition, future research should focus on questions regarding why some adolescents demonstrate durable control of their disease and others do not[144], and why T2DM is a more aggressive disease in adolescence compared to adulthood. Such studies will help to improve the overall understanding of youth T2DM as well as screening, prevention, and treatment strategies for such patients.

CONCLUSION

Pediatric T2DM is still a rare disease but recent reports indicate an increasing prevalence around the world, possibly following the increasing prevalence and severity of obesity in children and adolescents. Despite extensive research in the field over the last two decades, many knowledge gaps remain regarding the optimal management of obese children and adolescents with T2DM. The current approach is based on lifestyle interventions, including of diet and physical activity, on one hand, and pharmacologic treatment with metformin, insulin, and liraglutide in various combinations, on the other. What is important for everyone to realize, though, is that T2DM is a largely preventable disease if we manage to tackle its major risk factor, which is obesity. If families, schools, physicians, health services, policy makers and society altogether accept the obese child as the new "normal" and do not act promptly, no management approaches will be able to protect the next generation from many years of serious health problems and low-quality life.

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REVIEW

Maternal obesity as a risk factor for developing diabetes in offspring: An epigenetic point of view

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Abstract

According to the developmental origin of health and disease concept, the risk of many age-related diseases is not only determined by genetic and adult lifestyle factors but also by factors acting during early development. In particular, maternal obesity and neonatal accelerated growth predispose offspring to overweight and type 2 diabetes (T2D) in adulthood. This concept mainly relies on the developmental plasticity of adipose tissue and pancreatic β-cell programming in response to suboptimal *milieu* during the perinatal period. These changes result in unhealthy hypertrophic adipocytes with decreased capacity to store fat, low-grade inflammation and loss of insulin-producing pancreatic β-cells. Over the past years, many efforts have been made to understand how maternal obesity induces long-lasting adipose tissue and pancreatic β-cell dysfunction in offspring and what are the molecular basis of the transgenerational inheritance of T2D. In particular, rodent studies have shed light on the role of epigenetic mechanisms in linking maternal nutritional manipulations to the risk for T2D in adulthood. In this review, we discuss epigenetic adipocyte and β-cell remodeling during development in the progeny of obese mothers and the persistence of these marks as a basis of obesity and T2D predisposition.

Key Words: Development; Epigenome; Obesity; Type 2 diabetes; Adipose tissue; Beta cells

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Core Tip: According to the developmental origin of health and disease concept, maternal obesity and neonatal accelerated growth predispose offspring to metabolic diseases. White adipose tissue and pancreatic β-cells are key targets of developmental programming, although the underlying mechanisms remain elusive. Human and rodent studies have contributed to decipher the role of epigenetic mechanisms in the transgenerational inheritance of obesity and type 2 diabetes (T2D). In this review, we discuss the current understanding of the link between obesogenic maternal nutritional environment, developmental epigenetic adipocyte and β-cell remodeling and predisposition to obesity and T2D later in life.

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INTRODUCTION

Obesity and related metabolic diseases have doubled since 1980 and reached epidemic proportions over the past decades[1]. Obesity and expansion and dysfunction of white adipose tissue (WAT) are major drivers of type 2 diabetes (T2D), through induction of insulin resistance[2]. Insulin resistance is observed locally in hypertrophic adipocytes long before glucose intolerance develops[3]. The inability of further WAT expansion accelerates fat spillover, leading to ectopic fat deposition in skeletal muscle and liver and insulin resistance in those tissues[4,5]. Chronic inflammation in WAT is also considered a crucial causal factor for the development of insulin resistance and T2D in obese individuals[6,7]. Indeed, obesity-induced WAT remodeling provides intrinsic signals capable of initiating a local inflammatory response that may spread into the circulation, resulting in systemic insulin resistance and T2D[8]. An obesogenic environment and lipotoxic conditions also result in dysfunction and loss of insulin-producing pancreatic β-cells due to dedifferentiation, transdifferentiation, or death. These phenotypic alterations ultimately hamper insulin secretion[9].

Although genetics account for the variation in body weight and T2D, the dramatic rise in their incidence cannot be solely explained by genetic predisposition. Hence, environmental factors such as overnutrition, sedentary lifestyle, xenobiotics, and chemical exposure appear to be major contributors to the rapidly increasing prevalence[1]. In particular, studies in both humans and animal models suggest that excess nutrient supply in the fetal or neonatal period result in long-term programming of body weight set-point and predispose individuals to obesity and T2D[10-12]. Thus, the developmental origin of obesity and T2D perpetuate the vicious cycle of metabolic diseases across generations[12]. Among different mechanisms of transmission, epigenetics has emerged as a very important determinant[13]. In this review, we will present data on how maternal obesity programs T2D risk *via* epigenetic mechanisms by focusing on changes in WAT and β-cell physiology.

HERITABILITY OF OBESITY AND T2D: THE DEVELOPMENTAL ORIGIN OF HEALTH AND DISEASE CONCEPT

The developmental origin of health and disease concept (DOHaD) proposes a link between environmental challenges during early stages of growth and predisposition to metabolic disorders later in life. In particular, this concept states that suboptimal nutritional environment (*i.e.*, under or overnutrition) during the perinatal period can program or imprint the development of key tissues that play a central role in regulating energy homeostasis. Later in life, it might permanently determine physiological responses and ultimately produce energy balance dysfunction and metabolic diseases, such as obesity and T2D[10].

Originally called the Barker hypothesis or fetal programming, this concept arises

from epidemiological studies. Indeed, David Barker was the first to report that intrauterine growth retardation (IUGR) and low birth weight were associated with increased risk of metabolic syndrome-related diseases during adulthood[10]. As illustrated by the Dutch famine of 1944-45, offspring of mothers exposed to the famine presented with low birth weight associated with an increase in the incidence of dyslipidemia, obesity, and T2D later in life[13]. More recently, adults born during the Chinese famine of 1959-61 were also predisposed to overweight and T2D, constituting a major contributor to China's current T2D epidemic[14]. David Barker proposed the notion of a "thrifty phenotype," which placed an emphasis on development, arguing that nutritionally inadequate conditions in pregnancy not only affected fetal growth but also induced permanent changes in insulin secretory capacity and in glucose metabolism[15]. In humans with low birth weights, postnatal hypercaloric nutrition, and more specifically rapid catch-up growth, are also important accelerators in the etiology of adult-onset diseases[16]. As stated by the predictive adaptive response concept, the degree of mismatch between the pre-and postnatal environments is the key paradigm in developmental metabolic programming[17].

This concept has evolved from undernutrition to overnutrition. As shown in Figure 1, epidemiological and clinical studies have reported that individuals exposed to maternal overnutrition and/or obesity during pregnancy and lactation are also predisposed to increased risk of metabolic syndrome-related diseases later in life[18]. Subsequent meta-analyses have highlighted birth weight as a predictor of obesity and T2D. A U-shaped curve was proposed to explain the relationship between birth weight (a marker of fetal nutritional exposure) and the propensity to develop obesity in adulthood. Hence, it is currently well accepted that individuals born small or large (*i.e.*, low or high body fat percentage) have similar increased risks of obesity and related diseases later in life[19]. Over the past decades, animal studies have confirmed that maternal obesity during gestation and lactation, gestational diabetes, and accelerated growth of neonates predispose offspring to obesity and T2D[20,21].

Two main questions arise about the DOHaD concept. First, what is the basis of the persistent cellular memory of a developmental event, even when the initial stimulus has disappeared and despite continuous cellular turnover? Second, how two opposite maternal nutritional manipulations (under- *vs* overnutrition) may result in similar outcomes in adult offspring. Little is known about the cellular and molecular mechanisms underlying the phenomenon known as developmental programming. Among them, epigenetic modifications are likely to play a key role in the heritability of obesity and T2D[12,22,23].

EPIGENETIC MECHANISMS AS A BASIS OF THE DOHaD CONCEPT

Epigenetics can be defined as somatically heritable states of gene expression resulting from changes in chromatin structure without alterations in the DNA sequence[24]. Epigenetic modifications are transmitted from one cell generation to the next (mitotic inheritance) and can also be transmitted across generations (meiotic inheritance)[25]. These processes include DNA (hydroxy) methylation, histone post-translational modifications (PTMs) such as acetylation, phosphorylation, ubiquitination, and sumoylation, and noncoding RNA that regulates gene expression at both transcriptional and post-transcriptional levels[26]. DNA methylation, which results from the transfer of a methyl group, by DNA methyltransferase (DNMT), takes place at cytosines, mainly in the CpG islands, to form 5-methylcytosine (5mC). It serves to establish long-term gene silencing[27]. 5-hydroxymethylcytosine (5hmC) is another important cytosine modification catalyzed by the enzymes of the ten-eleven translocation methylcytosine dioxygenase (TET) family[28]. It serves as an intermediate for demethylation of 5hmC and is enriched in active transcriptional regulatory regions. Based on the histone code hypothesis, PTMs play crucial roles in controlling gene expression by adapting the local chromatin architecture and accessibility, allowing the recruitment of partners that modulate the transcriptional machinery. For example, acetylation of histone H3 lysine residues (H3Kac) and methylation of H3K4 (H3K4me1/3) are associated with active transcription while methylation of H3K9 (H3K9me3) generally indicates silenced chromatin. These histone PTMs are catalyzed by various enzymes including histone acetyltransferase (HAT), histone deacetylase (HDAC), histone methyltransferases (MTs)/demethylases[29,30]. Thus, the term epigenome refers to the combination of all chromatin modifications (*i.e.*, DNA methylation and PTMs) of a given cell type in an individual.

Histone-modifying enzyme activity is sensitive to cellular energy status and

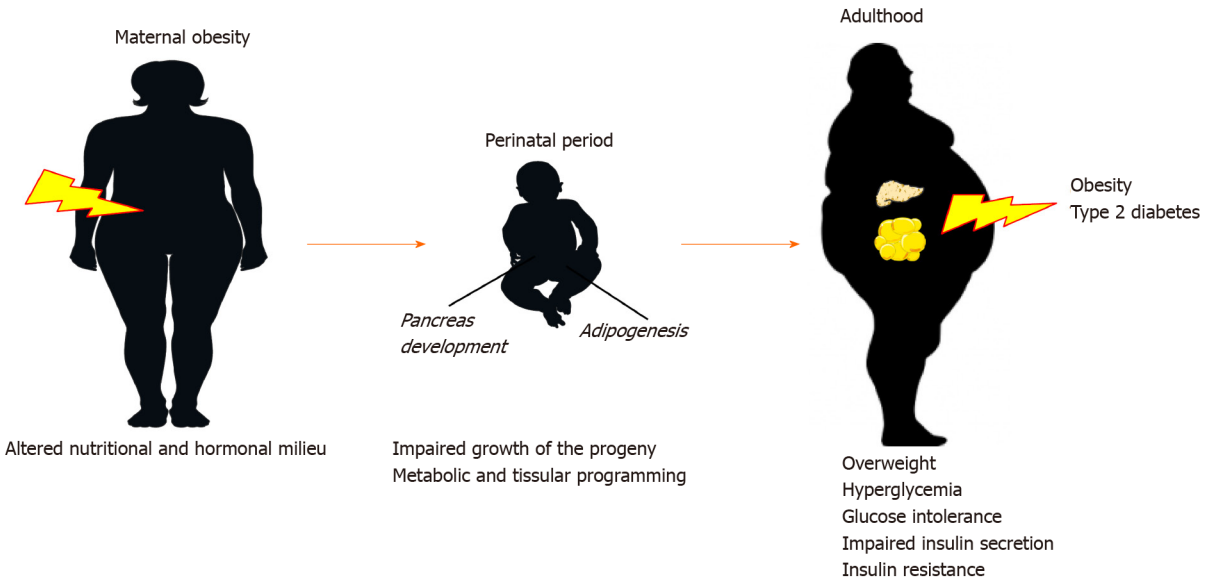

Figure 1 Maternal obesity and the developmental origin of health and disease concept. Maternal obesity results in impaired growth of offspring during fetal and perinatal period as well as metabolic and tissue programming. These developmental changes may have long-term consequences in the susceptibility to obesity and type 2 diabetes later in life.

hormonal response. In particular, it is highly dependent on intermediary metabolites that act as enzyme cofactors[6,31]. For example, HATs use acetyl-coenzyme A (CoA), histone MTs use S-adenosyl methionine (SAM), HDACs can use nicotinamide adenine dinucleotide, and histone demethylases can use flavin adenine dinucleotide or symbol-ketoglutarate as coenzymes[29,30]. Interestingly, sirtuin 1 is a nutrient-sensing HDAC and is associated with the risk of metabolic syndrome including T2D[32]. Hence, modifications of nutritional and hormonal milieu in offspring from obese dams may affect the developmental program of adipocyte and β-cells by impairing chromatin remodeling activities and DNA methylation[30]. Several studies have also reported the role of noncoding RNAs in WAT and pancreas development as well as in adipocyte and β-cell differentiation and function[33,34].

However, here we emphasize DNA methylation and chromatin modifications in progenitor cells, whose inappropriate editing during gestation and lactation may serve as a deleterious memory of exposure to a maternal obesogenic environment. The persistence of these marks throughout life and across generations may program permanent changes in gene expression and may account for inheritance of metabolic diseases.

DEVELOPMENTAL ORIGIN OF ADIPOSITY AND T2D

Based on the DOHaD concept, there are two main reasons why adipocyte and β-cells are key targets of perinatal programming. First, numerous studies have shown that manipulation of epigenetic machinery can alter cell fate and identity as well as cell-type-specific gene expression during both adipogenesis[33] and β-cell neogenesis (*i.e.*, adipocyte and β-cell formation, respectively)[35-41]. Epigenetic mechanisms are also known to play a crucial role in the control of β-cell identity and plasticity (*i.e.*, dedifferentiation and transdifferentiation) in adulthood[42-45]. Second, adipogenesis and β-cell neogenesis occur primarily during the second part of gestation in rodents, accelerate during early postnatal life, and remain active after weaning. The deleterious effects of maternal obesity operate during periods of development in which precursor cells are plastic (*i.e.*, possess the flexibility to adapt to the changing microenvironment) and where epigenetic remodeling is particularly dynamic and sensitive to the nutritional and hormonal milieu[12,19,46,47]. Hence, disturbance of the developmental program might result in severe dysfunctions in fetal pancreatic β-cells and adipose tissue and a profound perturbation of systemic glucose homeostasis in adulthood.

Adipose tissue development

WAT exists in multiple locations in the body and has two major subtypes, visceral and subcutaneous[12,48]. Unlike visceral WAT (vWAT), the metabolic plasticity of subcutaneous WAT (sWAT) is associated with increased insulin sensitivity and decreased occurrence of T2D[49]. In rodents, adipogenesis is particularly active during the perinatal period. These processes occur primarily during the last week of gestation (the first fat cells appear between the fourteenth and the eighteenth days of gestation)[50-52] and accelerate during early postnatal life until pups are weaned. However, sWAT develops during late gestation and lactation whereas vWAT formation is mainly initiated after birth[50]. The developmental origin of adipocytes remains elusive. Cell lineage tracing, adipocyte precursor fate studies and subsequent molecular analysis demonstrated a heterogeneity of the adipose lineage between fat depots, but also within individual adipose depots, in terms of cell origin, spatio-temporal adipogenic potential, gene expression profile, growth rate, and biological properties. Hence, the ability of adipose precursors to differentiate during adipogenesis is dependent on the anatomical location and the local microenvironment, leading to the concept of depot-specific adipogenesis[53,54].

Adipocytes are derived from multipotent mesenchymal stem cells (MSCs), which are first committed to the adipogenic lineage and then transformed into preadipocytes. This phase is then followed by terminal differentiation during which preadipocytes become mature adipocytes that develop the ability to store lipids in a large monolocular lipid droplet and display endocrine properties. The process of adipocyte differentiation involves three defined steps. The first step is the commitment of MSCs to the adipocyte lineage. The second step is mitotic clonal expansion involving DNA replication and duplication of cells. The third step is terminal differentiation, which involves transcriptional factors such as CCAAT-enhancer-binding proteins (C/EBP) , β and δ, peroxisome proliferator-activated receptor γ (PPARγ), and significant expression of adipocyte-specific genes such as adiponectin and leptin[55-58].

Our understanding of adipogenesis comes mainly from studies using preadipocyte cells such as 3T3-L1[33,59]. Adipogenesis involves a dynamic reorganization of the chromatin landscape at specific developmental stages that is associated with the recruitment of multiple transcription factors controlling the expression of adipocyte-specific genes. This process is regulated through the remodeling of cell-specific histone marks and DNA (hydroxy)methylation[60,61]. Thus, in undifferentiated adipocytes, the master adipogenic transcription factors, zinc finger protein 423 (ZFP423), C/EBPβ, and PPARγ, are in a poised state owing to the bivalent presence of the active H3K4me3 and repressive H3K9me3 marks in their promoters[59]. ZFP423 was identified as crucial for adipogenesis by promoting PPARγ expression[62]. During the early phase of adipocyte differentiation, the reorganization of the chromatin structure and chromatin opening along with the recruitment of several early transcription factors, such as C/EBPβ/δ, coincide with the removal of repressive histone marks (*i.e.*, H3K9me3) and the enrichment of active chromatin marks, including H3K27ac, and H3K4me3, as well as DNA hydroxymethylation, in their promoters[60]. During differentiation, chromatin remodeling primes genomic regions to allow the expression of CEBPα and PPARγ, their specific binding on the chromatin as well as their target genes[60]. For instance, H3K4 MT mixed-lineage leukemia (MLL) 3 and MLL4 are recruited by C/EBPβ to activate the enhancers of C/EBPα and PPARγ and induce C/EBPα and PPARγ expression during adipogenesis. Once induced, C/EBPα and PPARγ recruit MLL3/MLL4 to further activate enhancers of downstream target genes[63].

Beta cell development

The pancreas is derived from the ventral and dorsal endoderm, which stepwise differentiate into exocrine and endocrine lineages. Most of our current understanding of neogenesis is derived from rodent studies. In the developing pancreas, cell fate commitment towards specific endocrine subtypes is controlled by a complex orchestrated expression of specific transcription factors. In rodents, pancreas development undergoes two transitionary periods, a first wave transition (from embryonic days E9.5-E12.5) during which the endocrine cells that are formed are primarily -cells or multi-hormonal cells and a second wave transition (from E12.5 to birth), which is the main period of endocrine cell formation including β-cells[34,64-66].

Pancreatic endocrine cells arise from multipotent endocrine progenitor-precursors (EPs) that express PDX1. The master regulator of EP formation and differentiation is Ngn3, which is indispensable for endocrine cell formation. Higher expression levels of Arx and Pax4 favor formation of - and β/δ-cells, respectively. Differentiation toward β-cell fate depends on the expression of several other transcription factors such as

FOXA2, NKX6-1, NEUROD1, NKX2-2 and MAFA which are important for the establishment and maintenance of β-cell identity. In rodents, embryonic β-cells appear during the perinatal period, are immature, highly proliferative, and plastic but respond poorly to glucose stimulation[67,68]. After birth, β-cells follow a biphasic pattern of maturation[69]. The first 2 wk of life define a first maturation wave characterized by active proliferation that results in an increase in the β-cell mass, a characteristic that is progressively lost. The second wave of maturation coincides with the third week of life and the weaning period[70]. During that time, β-cells differentially regulate metabolic pathways and acquire physiological functions, such as glucose-stimulated insulin secretion in response to extracellular glucose[71,72]. In rodents, the postnatal maturation of β-cells is driven by weaning (dietary change from high-fat milk to high-carbohydrate chow), and studies have suggested that microRNAs (miRNAs) have a central role in regulating postnatal β-cell maturation[70,73].

Several studies support the notion that the development and heterogeneity of EPs are regulated at the chromatin level. Our understanding of epigenetic mechanisms involved in β-cell formation and maintenance of identity comes from *in vitro* differentiation protocols of pluripotent stem cells[74] and studies using inhibitors of epigenetic enzymes or employing mice deficient models for different classes of epigenetic modifiers. First, the use of HDAC inhibitors on embryonic pancreas explants resulted in increased numbers of endocrine progenitors and β-cells whereas embryonic pancreatic overexpression of HDAC reduced the β-cell mass[35]. Generation of HDAC5- or -9-deficient mice increased the insulin-secreting β-cell mass[36,44]. Second, deletion of Jmjd3, a histone demethylase for the repressive H3K27me3 mark at the pancreatic progenitor stage, impaired the efficiency of endocrine cell fate transition and subsequent islet formation in mice[38]. Third, numerous genes critical for β-cell function that are bivalently marked in -cells by both active H3K4me3 and repressive H3K27me3 histone modifications are monovalently marked by active H3K4me3 in β-cells. This bivalency suggests a plastic epigenetic state for key β-cell genes in α-cells indicating a paused state with potential for activation[45]. To maintain β-cell identity, genes important for -cell function have to be actively repressed by the DNA methy-transferases DNMT1 and DNMT3a in β-cells, suggesting that repression of the cell program (*i.e.*, methylation of the ARX promoter) is necessary for β cell identity to be maintained[40]. Recent studies have also shown that inhibiting DNA methylation in pancreatic progenitor cells promotes α-cell production[39]. In addition, the hypermethylation of CpG islands can reduce the expression of *Hnf4a* (hepatocyte nuclear factor 4) gene and affect the differentiation of β-cells[75]. Four, the β-cell-specific deletion of embryonic ectoderm development, a component of the polycomb repressive complex 2 (PRC2) in mice results in β-cell dysfunction, dedifferentiation, and diabetes development associated with chromatin-state-associated transcriptional dysregulation[76]. Of note, PRC2 methylates the histone lysine residue H3K27, especially H3K27me3 that acts to silence gene expression[77]. β*Eed*^{KO} cells exhibited reduced levels of the mature β-cell markers Pdx1, MafA, Nkx2-2, and Nkx6-1, and thus loss of β-cell identity. At the same time, they upregulated immature β-cell and progenitor-specific genes[76]. The finding of the role of loss of PRC2 activity in β-cell plasticity suggests that maintaining proper and specific histone marks and chromatin state at precise loci is crucial to maintain normal β-cell development and functionality.

PROGRAMMING MECHANISMS OF OBESITY AND T2D

Adipose tissue programming

Clinical observations among human and animal studies have determined two major determinants for metabolic health and insulin sensitivity. On one hand, the ability of sWAT to store excess fat (*i.e.*, storage capacity) rather than allowing it to accumulate in ectopic depots such as liver, muscle and vWAT, is of prime importance. On the other hand, the ability to recruit and differentiate new adipocytes (*i.e.*, activation of adipogenesis resulting in increased storage capacity) in sWAT also reduces risk of metabolic diseases including T2D in overweight individuals[78]. In obesity, WAT expands either by hyperplasia (increase in adipocyte number) or hypertrophy (increase in adipocyte size), where the latter is associated with insulin resistance and inflammation.

Numerous studies have shown that maternal obesity modifies the expansion capacity of WAT in offspring throughout life. In particular, the activity of key adipogenic transcription factors was impacted by maternal obesity during development, leading to impaired expandability of WAT in offspring[46,47,79]. One of the

most well-studied mechanisms is the modulation of *Zfp423* gene expression and activity in rodents. *Zfp423* expression defines committed preadipocytes, and its expression persists throughout adipocyte differentiation[62,80]. Inactivation of *Zfp423* during WAT development results in arrested differentiation, specifically of sWAT[81]. As a key developmental gene, *Zfp423* promoter has a bivalent region with enrichment of both H3K27me3 and H3K4me3 histone marks[82]. Offspring of obese mice were overweight, with an increased fat mass that was correlated with persistently elevated *Zfp423* activity in WAT[83]. Developmental epigenomic remodeling of these marks in the *Zfp423* promoter accounts for persistent higher *Zfp423* expression later in life. During the second part of gestation (E14.5) in which adipogenic activity was elevated, the H3K27me3 histone mark and DNA methylation were lower in the *Zfp423* promoter, whereas the H3K4me3 histone mark was higher in the fetuses of obese dams[83]. At weaning, WAT neonates still showed elevated *Zfp423* activity and exacerbated adipogenesis resulting in increased numbers of adipocytes and adiposity[83-85]. Overweight adult mice showed persistent increased gene expression with DNA hypomethylation in the *Zfp423* promoter despite impaired hyperplasia when fed a high-fat (HF) diet[84].

A possible interpretation is that individuals from obese mothers with dysfunctional WAT (*i.e.,* hypertrophic adipocytes), inflammation, and insulin resistance displayed a failure of WAT plasticity and inappropriate expansion of the adipose progenitors in sWAT. This might be due to maternal obesogenic environment and premature exhaustion of the stock of resident adipocyte progenitors during development that favors hypertrophy *vs* hyperplasia to store excess energy later in life[12]. In line with these findings, human MSC from the umbilical cords of infants born to obese mothers exhibit a greater potential for adipogenesis[86]. Godfrey *et al*[87], also reported a strong association between methylation of the retinoid-X-receptor α (RXRα) promoter region from DNA extracted from the umbilical cord of infants born to mothers with low carbohydrate intake during pregnancy and the degree of adiposity 6-9 years later. High methylation in the *RXRa* promoter reduces gene expression and alters insulin sensitivity and glucose metabolism in differentiated adipocytes[88].

Another well-studied epigenetic mechanism is the modulation of *Pparγ* expression and activity in offspring of obese dams. The regulation of *Pparγ* expression in WAT *via* DNA methylation and histone modification at its promoter region is well illustrated[89-91]. Decreased *Pparγ2* expression in the WAT of adult rat offspring is generally associated with tissue dysfunction[92]. Several studies suggest that the reduction in *Pparγ2* expression may be due to epigenomic remodeling occurring during WAT development. In weanling rats from obese dams, reduced *Pparγ2* mRNA levels were observed together with DNA hypermethylation and decreased enrichment of H3ac and H3K4me3 active marks in the *Pparγ2* promoter region. In adulthood, DNA hypermethylation of the *Pparγ2* promoter and the reduction of *Pparγ2* mRNA expression levels were still observable[92]. It is tempting to speculate that the decreased expression of the master regulator of adipogenesis and lipid storage is an adaptative mechanism to avoid further deleterious adipocyte hypertrophy[93]. Interestingly, adult offspring from dams fed an HF diet only during lactation were predisposed to obesity, with increased expression of stearoyl-CoA desaturase 1 (SCD1), a key enzyme of lipid storage. Higher *Scd1* gene expression was associated with reduced DNA methylation in the *Scd1* promoter surrounding a *Pparγ*-binding region[94].

Maternal obesity also predisposes to the development of a chronic low-grade proinflammatory state associated with insulin resistance in WAT offspring[12,95,96]. Several studies showed that elevated proinflammatory adipocytokine production such as leptin, WAT inflammation, and macrophage infiltration can be transmitted across generations *via* epigenetic mechanisms. For instance, obesity-prone offspring from obese rats displayed elevated leptin gene expression, hyperleptinemia, and adipocyte hypertrophy in WAT[79,97]. During lactation, increased *leptin* gene expression arises from higher DNA hydroxymethylation and active histone H3K4me1/H3K27ac marks in an enhancer region[97]. These histone marks persisted in the WAT of adult offspring in hypertrophic adipocytes[97]. Maternal obesity in mice also results in persistent hypermethylation of the. H4K20 histone mark in the promoter region in offspring that persists across generations[98-100]. Interestingly, multigenerational HF diet feeding in female mice resulted in gradually increased WAT weight, proinflammatory markers, and immune cell infiltration associated with a gradual decrease in DNA methylation of inflammation-associated genes (*i.e.,* Toll-like receptors) in WAT across generations (up to F2)[12]. However, the effects of a maternal HF diet *vs* maternal obesity on offspring WAT inflammation and glucose homeostasis remain to be determined[12,101].

It is interesting to note that programmed changes in miRNAs may also account for both adipose tissue expandability and insulin resistance in offspring from

malnourished dams. On the one hand, an increase in miR-483 and parallel reduction in growth differentiation factor 3 have been reported in WAT from the offspring of dams fed a low protein (LP) diet[102]. That may lead to a reduction in the expandability of WAT, and therefore, increased ectopic fat deposition. Similar observations were also described in WAT from individuals with low birthweights, showing conservation of this programmed mechanism. On the other hand, maternal obesity resulted in higher miR-126 levels in WAT of mice offspring which led to reduced expression of key insulin signaling proteins, including insulin receptor substrate-1[103,104].

β-cell programming

The pancreas is an organ that is particularly sensitive to nutritional imbalance during intrauterine organogenesis[34]. Rodent models have been mainly used to investigate the effects of maternal obesity on islet development and function and to decipher underlying programming mechanisms. In rodent models, maternal obesity results in decreased β-cell mass and insulin secretion at birth[105]. Islets from offspring born to obese or HF diet-fed mothers have decreased pancreatic insulin content, *Pdx1* expression in adult islets[106,107] with remodeling of the architecture of the islets, characterized by an increase of α-cells in the centers of pancreatic islets[108,109]. Interestingly, pancreatic β-cell dysfunctions occur in offspring from obese dams in a sex-dependent manner. Very few data are available regarding the effects of maternal obesity on the β-cell programming in offspring in terms of epigenetics. However, consistent with the DOHaD concept, several studies highly suggest that maternal nutritional manipulations have a transgenerational influence on β-cell development and function through long-lasting effects on the offspring epigenome, predisposing to T2D later in life.

The first evidence comes from a rat model of IUGR caused by bilateral uterine artery ligation leading to a lower body weight at birth[110]. This deficient intrauterine environment affects fetal development through progressive and permanent dysregulation of gene expression and function of β-cells resulting in the development of T2D. For instance, adult IUGR rats exhibited a persistent reduction of Pdx1 expression levels in β-cells associated with chromatin remodeling throughout development. In the fetus, prior to the onset of T2D, deacetylation of histones H3 and H4 and recruitment of *Hdac1* in the promoter of *Pdx1* were associated with decreased mRNA expression levels. Loss of acetylation was accompanied by loss of binding of the key transcription factor Usf-1. In neonates, the active histone H3K4me3 mark was lower, and the repressive histone H3K9me2 mark was higher, at the hypomethylated Pdx1 promoter of IUGR islets. Once T2D occurs in adulthood, the promoter was hypermethylated, resulting in permanent silencing of the *Pdx1* gene[111]. To achieve a more complete picture of DNA methylation changes, Thompson *et al*[112] have generated a whole DNA methylation map of the rat genome in IUGR pancreatic islet cells. They showed that IUGR changes cytosine methylation at approximately 1400 loci in IUGR rats before the onset of diabetes. Interestingly, epigenetic dysregulation occurred preferentially at intergenic sequences close to genes regulating cellular processes that were impaired in IUGR islets, including β-cell proliferation, insulin secretion, and apoptosis. The modifications of the DNA methylation were associated with changes in mRNA expression levels[112]. Consistent with this notion, early postnatal overnutrition (newborns suckled in a small litter) accelerates aging-associated epigenetic DNA hypermethylation in dysfunctional pancreatic islets of weaned and adult offspring[113].

Data obtained from a rat model of maternal protein restriction (LP) during pregnancy and lactation that resulted in IUGR reinforce this hypothesis. Maternal LP altered the pancreatic structure, islet areas and quantities and resulted in abnormal morphological changes during pancreatic development[114]. LP offspring had normal glucose tolerance in young adult life but suffered from an age-dependent loss of glucose tolerance and developed T2D in adulthood[115]. The authors showed that the *Hnf4a* gene encoding a transcription factor required for β-cell differentiation, and which has been implicated in the etiology of T2D, is epigenetically regulated by maternal LP diet and aging in rat islets[116]. IUGR offspring have progressive epigenetic silencing at the promoter-enhancer regions (*i.e.*, decreased active H3ac and H3K4me1/3 and increased repressive H3K9me2 and H327me3 histone marks), which weakens their interaction and results in a permanent reduction in *Hnf4a* expression[116]. It is interesting to note that changes in DNA methylation also take place in the pancreatic islets of mice born to mothers with gestational diabetes mellitus. In the offspring, hypermethylation of the imprinted *Igf2/H19* (insulin-like growth factor-2) locus in pancreatic islets may account, at least in part, for impaired islet ultrastructure and function that has been shown to be transmitted to subsequent generations[117].

Although precise mechanisms linking offspring β-cell programming and maternal obesity are lacking, it is tempting to speculate that the developmental pathways of pancreatic endocrine lineages could be epigenetically reprogrammed through similar mechanisms, ultimately resulting in impaired β-cell number and plasticity.

INTER- AND TRANSGENERATIONAL INHERITANCE OF OBESITY AND T2D

Growing evidence suggests that epigenetic dysregulation of key metabolic genes implicated in adipocyte and β-cell development and function in offspring contribute to developmental programming of T2D[118]. As summarized in Figure 2, chromatin remodeling and changes in DNA methylation may account, at least in part, for the molecular basis of intergenerational effects. The establishment of epigenetic marks on somatic stem cells will give rise to mature adipocytes and β-cells of the fetus (F1) as well as the germline of the fetus (the future F2). The persistence of these marks into adulthood may program obesity-associated insulin resistance up to F2. Consistent with this notion, maternal obesity before and throughout pregnancy and lactation results in altered development of the pancreas in F1 and F2 mouse offspring[119]. However, it is less clear so far how developmentally induced epigenetic modifications may persist beyond the F2. Only persistent phenotypes in the F3 and subsequent generations represent true transgenerational epigenetic inheritance, as they are stably transmitted through the F2 germline, which is not directly exposed to the initial maternal nutritional insult[120,121]. While the mitotic heritability of epigenetic marks is widely accepted, the existence and role of transgenerational epigenetic inheritance in mammals remain controversial. Most of our knowledge concerning germ cell formation in mammals comes from mouse models. In mice, primordial germ cells undergo a global epigenetic remodeling during germline development and following fertilization resulting in a complete resetting of the epigenetic memory arising from the parents and the establishment of sex-specific gamete identity. This event limits the stable transmission of epigenetic marks acquired during development or imposed by the environment from one generation to the next. However, In rodents, it has been clearly demonstrated that the epigenetic states are not entirely reprogrammed. Some imprinted genes escape demethylation processes resulting in intergenerational inheritance[122].

Increasing evidence suggests that epigenetic modifications of the sperm and the spermatozoa are key players in transgenerational epigenetic inheritance in subsequent F2 male generations and beyond[98,123-127]. Indeed, a significant increase in body size, adiposity and reduced insulin sensitivity were reported in F1 and F2 after maternal obesity through both maternal and paternal lineages[128]. However, in the F3 generation, those metabolic alterations were only displayed by females and only *via* paternal lineage in the absence of any further nutritional stimulus[129,130]. Although the implication of DNA methylation and histone modifications cannot be totally excluded, noncoding RNAs have emerged as an alternative mode of transgenerational epigenetic inheritance from the male germline[129-131].

To our knowledge, there are very few data providing evidence for transgenerational epigenetic inheritance in humans. Human epidemiological studies suggest that grandparental overnutrition increases the rates of diabetes and cardiovascular diseases risk in F2[132]. Increased risk for obesity and related metabolic diseases was observed in children whose parents were of normal weight but whose grandparents were obese[133].

CONCLUSION

Consistent with the DOHaD concept, epigenetic research conducted on inter- and transgenerational inheritance of obesity and T2D has shed light on new molecular mechanisms. An important challenge for the scientific community is that the solutions to the transmission of obesity and T2D beyond the scope of health system prevention programs. In this context, a better understanding of the underlying mechanisms involved in the epigenetic regulation of adipocyte and β-cells programming becomes a necessity. Of high interest, deciphering the epigenetic mechanisms leading to enhanced β-cell mass, β-cell proliferation, and function defects during T2D should guide toward the identification of novel therapeutic targets.

The reversible nature of epigenetic modifications, together with recent advances in

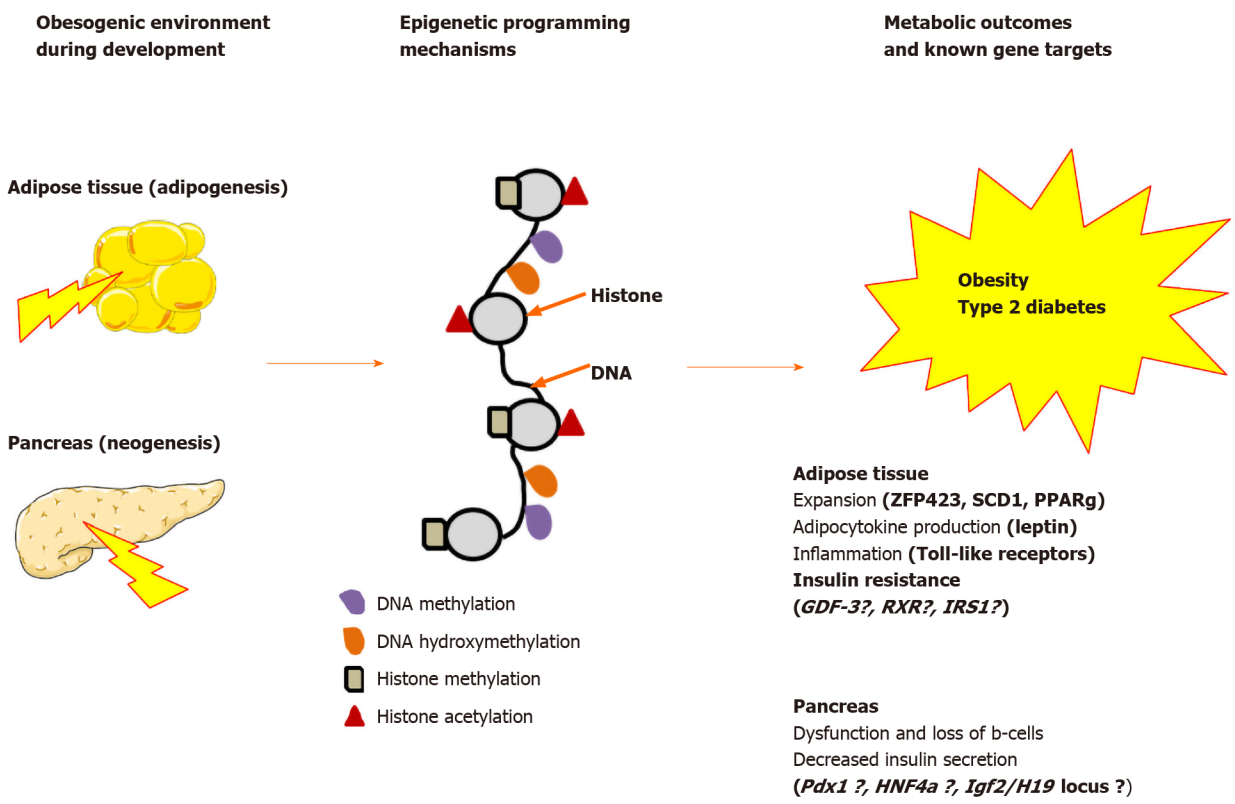

Figure 2 Epigenetic programming mechanisms and offspring gene targets. Gene targets with epigenetic modifications in the offspring of obese mothers are indicated. Gene targets indicated with a question mark have been evidenced by maternal nutritional manipulations other than maternal obesity. ZFP423: Zinc finger protein 423; SCD1: Stearoyl-coenzyme A desaturase 1; PPAPγ: Peroxisome proliferator-activated receptor γ; GDF-3: Growth differentiation factor 3; RXR: Retinoid-X-receptor; IRS1: Insulin receptor substrate-1; HNF4α: Hepatocyte nuclear factor 4 α; Igf2: Insulin-like growth factor-2.

epigenome-targeting methods, provide a new opportunity for alternative epigenetic therapies[134,135]. Indeed, targeting epigenetic machinery during early development is an attractive way to reduce adverse outcomes of maternal obesity. On one hand, it is crucial to better understand when and how an obesogenic environment may affect the fate of stem cells during adipocyte and β-cell development *via* epigenetic mechanisms. In the near future, determining the nature and kinetics of recruitment of enzymes controlling the PTMs of histones and DNA methylation involved in the complex transcriptional program will be needed. High-throughput DNA sequencing approaches in epigenomics for genome-wide profiling of global DNA methylation and histone modifications should allow determining changes in chromatin landscape throughout development. As a follow-up, high-throughput CRISPR-Cas9 technologies for epigenome editing might allow efficient targeting of key epigenetic marks as therapeutic option[136].

On the other hand, the use of natural compounds or pharmacological agents leading to DNA methylation and histone modifications, such as DNMT inhibitors and HDAC inhibitors have been already validated as an innovative approach[132]. Dietary supplementation of methyl donors during perinatal period was found to alleviate the adverse consequences of maternal malnutrition[137,138]. For instance, pharmacological modulation of epigenetic enzymatic machineries *via* drugs to improve β-cell functionality has already been recognized as promising new avenue for future therapeutic purposes[135,139-141]. Most important, targeting transient and reversible epigenetic modifications during early stages of life, either by genetic or pharmacological means, provides a promising therapeutic way to counteract adverse programming effects on maternal obesity in the progeny.

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REVIEW

Diabetic heart disease: A clinical update

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Abstract

Diabetes mellitus (DM) significantly increases the risk of heart disease, and DM-related healthcare expenditure is predominantly for the management of cardiovascular complications. Diabetic heart disease is a conglomeration of coronary artery disease (CAD), cardiac autonomic neuropathy (CAN), and diabetic cardiomyopathy (DCM). The Framingham study clearly showed a 2 to 4-fold excess risk of CAD in patients with DM. Pathogenic mechanisms, clinical presentation, and management options for DM-associated CAD are somewhat different from CAD among nondiabetics. Higher prevalence at a lower age and more aggressive disease in DM-associated CAD make diabetic individuals more vulnerable to premature death. Although common among diabetic individuals, CAN and DCM are often under-recognised and undiagnosed cardiac complications. Structural and functional alterations in the myocardial innervation related to uncontrolled diabetes result in damage to cardiac autonomic nerves, causing CAN. Similarly, damage to the cardiomyocytes from complex pathophysiological processes of uncontrolled DM results in DCM, a form of cardiomyopathy diagnosed in the absence of other causes for structural heart disease. Though optimal management of DM from early stages of the disease can reduce the risk of

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diabetic heart disease, it is often impractical in the real world due to many reasons. Therefore, it is imperative for every clinician involved in diabetes care to have a good understanding of the pathophysiology, clinical picture, diagnostic methods, and management of diabetes-related cardiac illness, to reduce morbidity and mortality among patients. This clinical review is to empower the global scientific fraternity with up-to-date knowledge on diabetic heart disease.

Key Words: Diabetic heart disease; Type 2 diabetes mellitus; Type 1 diabetes mellitus; Coronary artery disease; Cardiovascular disease; Cardiac autonomic neuropathy; Diabetic cardiomyopathy

Core Tip: Cardiovascular disease is the major cause of morbidity and mortality among patients with diabetes mellitus (DM). Three distinct and common clinical entities viz., coronary artery disease (CAD), cardiac autonomic neuropathy (CAN) and diabetic cardiomyopathy (DCM) collectively form diabetic heart disease. The pathophysiological mechanisms involved in the development of diabetic heart disease are complex and involve multiple metabolic and molecular pathways. Although most clinicians are well-aware that CAD is a cardiac complication of DM, the awareness about CAN and DCM is remarkably low. This clinical update discusses the pathophysiology, diagnostic aspects, and management options for patients with diabetic heart disease, to empower clinicians across the globe to optimally manage the disease scientifically.

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INTRODUCTION

The current prevalence of diabetes mellitus (DM) is 463 million, which is equivalent to 9.3% of the world population. The global pandemic of diabetes is expected to raise this figure to 578 million (10.2%) by the year 2030 and 700 million (10.9%) by 2045[1]. This pandemic is associated with serious economic implications. Nearly 10% of the global health expenditure is spent on diabetes care, which is equal to United States $760 billion in 2019, and this is expected to reach United States $845 billion by the year 2045[2]. DM is the eighth leading cause of death and the third leading cause of years lost with disability[3]. Diabetes greatly increases the risk of cardiovascular diseases including ischaemic heart disease, stroke, and heart failure (HF). These cardiovascular complications contribute to a majority of diabetes-associated morbidity and mortality. Moreover, the healthcare cost related to diabetes management is predominantly contributed to by the expenditure for treatment of these complications. Therefore, its prevention is of paramount importance to reduce morbidity, mortality and healthcare costs[2].

Cardiac disease that develops as a direct consequence of DM in patients with type 1 DM (T1DM) or type 2 DM (T2DM) is known as diabetic heart disease. Diabetic heart disease is a conglomeration of coronary artery disease (CAD), cardiac autonomic neuropathy (CAN), and diabetic cardiomyopathy (DCM), and these diseases are characterized by molecular, structural, and functional changes in the myocardium[4]. CAD is the often-recognised cardiac complication of diabetes. On the contrary, CAN and DCM although common, are often undiagnosed complications with devastating consequences, including increased mortality. This review aims to critically appraise the available literature related to cardiac complications of DM, to empower clinicians to manage DM more judiciously and scientifically.

DM, CARDIOVASCULAR DISEASE, AND CARDIOVASCULAR MORTALITY

T1DM is associated with a twofold rise in all-cause mortality and a threefold rise in cardiovascular mortality, compared to the general population, even with optimal glycaemic control with glycosylated haemoglobin (HbA1c) of ≤ 6.9%/52 mmol/mol. On the contrary, T1DM patients with poor glycaemic control (HbA1c > 9.7%/83 mmol/mol) have an eightfold higher risk of all-cause mortality and a tenfold higher risk of cardiovascular mortality[5]. Among patients with T1DM, acute metabolic complications including hypoglycaemia and diabetic ketoacidosis are the leading causes of all-cause mortality in subjects under 30 years, whereas cardiovascular diseases (CVD) are the predominant causes of all-cause mortality in those over 30 years of age[6]. Overall, CVD contributes to nearly 44% of the all-cause mortality in T1DM subjects at any age[7].

In a recent observational study among adults with T1DM, the level of glycaemic control, hypertension, dyslipidaemia, and diabetic nephropathy were significantly associated with the risk of CVD in addition to body mass index, increasing age, and duration of diabetes[8]. T1DM subjects without nephropathy have a minimal increase in all-cause mortality, indicating that nephropathy is the main driver for CVD and all-cause mortality[9]. Apart from nephropathy, other microvascular complications of DM such as retinopathy and peripheral polyneuropathy are associated with adverse cardiovascular outcomes and all-cause mortality in T1DM[10].

In subjects with T2DM, nearly 52% of all-cause mortality is contributed by CVD[7]. In general, T2DM is associated with a twofold rise in all-cause mortality and a threefold rise in cardiovascular mortality. The relative risks are higher in women and subjects under 55 years of age, as compared to men and those above 55 years of age[11]. Similarly, the relative risks are also higher with poor glycaemic control and renal disease characterized by albuminuria and deterioration in estimated glomerular filtration rate. T2DM patients under 55 years of age with HbA1c of ≤ 6.9% (52 mmol/mol) have a twofold rise in all-cause mortality and cardiovascular mortality. Even with a modest rise of HbA1c to the range of 7.0%-7.8% (53-62 mmol/mol) there is a twofold rise in all-cause mortality and 2.5-fold rise in cardiovascular mortality; and when it is in the range of 7.9%-8.7% (63-72 mmol/mol), there is a 2.5-fold rise in all-cause mortality and a fourfold rise in cardiovascular mortality. With HbA1c in the range of 8.8%-9.6% (73-82 mmol/mol), there is a threefold rise in all-cause mortality and a fourfold rise in cardiovascular mortality; and in uncontrolled diabetes with HbA1c > 9.7% (83 mmol/mol), there is a fourfold rise in all-cause mortality and a fivefold rise in cardiovascular mortality (Swedish National Diabetes Register)[12]. This suggests adverse cardiovascular and all-cause mortality risks in relation to poorer glycaemic control in T2DM subjects.

Optimal glycaemic control and control of cardiovascular risk factors are associated with the attenuation of all-cause mortality and cardiovascular mortality in T2DM. A recent observational study showed a modest but significant reduction in mortality with improved diabetes care (only 16% excess all-cause mortality and 18% excess cardiovascular mortality compared to the figures mentioned above for those with poor control)[13]. Among patients with T2DM, men show a higher absolute risk of first-time cardiovascular complications like myocardial infarction (MI), stroke, cardiovascular mortality, and HF (major adverse cardiovascular events including HF), whereas T2DM women exhibit a higher relative risk of first-time cardiovascular complications. This could be explained by the fact that diabetes attenuates the protective effect of oestrogen on atherosclerosis. Other possibilities include differences in the cardiovascular risk burden between sexes, sex differences in accessing healthcare resources for primary prevention, poorer glycaemic control, or treatment adherence in women. However, the sex difference has no impact on recurrent cardiovascular events[14].

DM is a CAD equivalent

Diabetes mellitus was considered a '*CAD equivalent*' when earlier studies showed that patients with diabetes without prior MI have a risk of death from CAD equal to that of patients without diabetes, but with prior MI[15]. However, subsequent studies and a meta-analysis have proven that '*CAD equivalent*' is an overestimation, and there is a 43% lesser risk of developing CAD in subjects with diabetes without prior MI compared to those without diabetes but with prior MI[16]. A small coronary angiographic study showed that the cardiovascular complications that occur in T2DM patients depend on angiographic status rather than diabetes status, meaning that in the absence of obstructive CAD on angiography, there is little difference in the incidence of cardiovascular events among patients with or without diabetes[17].

A population-based study from Denmark stratified 93866 patients who underwent coronary angiography based on the presence or absence of diabetes and obstructive CAD. It was observed that among patients without significant CAD, those with or without diabetes have equivalent all-cause mortality, cardiovascular mortality, and MI[18]. The study also observed that among patients without significant CAD, those with diabetes were more often on prophylactic therapy with aspirin, statin, and antihypertensive agents as compared to those without diabetes. Thus, for patients with diabetes, prophylactic therapy could reduce the risk for MI and mortality equivalent to that of a person without diabetes.

DM AND CAD

The Framingham study observed that diabetes is associated with a 2-4 times greater risk for MI and 4-6 times greater risk for HF[19]. Cardiovascular complications including CAD and stroke are the causes of death in nearly 75% of patients with T2DM in developing countries[20]. The INTERHEART study supported the association between diabetes and MI on a global platform. With the implementation of appropriate primary prevention strategies, the risk for first-time cardiovascular complications has come down significantly. Similarly, with effective revascularisation techniques and secondary prevention strategies, the risk for recurrent cardiovascular events has significantly reduced[21].

Pathophysiology of CAD in DM

The phenomenon of persistent hyperglycaemia associated with increased cardiovascular disease is known as "metabolic memory" or "legacy effect". There are several extremely complex mechanisms involved in mediating this phenomenon (Figure 1). Advanced glycation end products (AGEs) are generated by nonenzymatic glycation of proteins, lipids, or lipoproteins. The triggers for AGEs generation are hyperglycaemia, hypoxia, ischaemia, or reperfusion[22]. AGE-Receptors for AGE (RAGE) interaction exerts pro-inflammatory effects, generates reactive oxygen species (ROS), expresses adhesion molecules in the endothelium including vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1), promotes entry of monocytes into the subendothelium, decreases vasodilation by decreasing nitric oxide (NO), enhances vasoconstriction by increasing endothelin-1, enhances macrophage phagocytosis by expressing the scavenger receptors (SR) on the surface of macrophages including cluster of differentiation-36 (CD36) and SR class A1[23,24].

Another mechanism by which diabetes increases the cardiovascular risk is the atherogenic modification of low-density lipoprotein (LDL), in which the LDL molecule is first desialylated to form small dense LDL. This is followed by oxidation or glycation of small dense LDL, which favours the interaction with subendothelial proteoglycan, enhancing the retention time of LDL, the LDL phagocytosis by the macrophages to form the lipid-laden foam cells, and the release of proinflammatory cytokines including tumour necrosis factor-alpha (TNF-α), interleukin 1-beta (IL1-β), IL-6, and matrix metalloproteinase (MMP) by the foam cells[23]. This in turn augments the atherogenic potential of LDL in subjects with DM, even at the normal levels for a nondiabetic individual.

Diabetes mellitus is associated with increased oxidative stress due to either increased production of ROS/reactive nitrogen species (RNS) or decreased clearance of ROS/RNS. Various triggers for ROS/RNS are direct effects from hyperglycaemia (excessive activation of mitochondrial electron transport chain), or indirect effects from AGEs, cytokines, upregulation of polyol pathway, upregulation of hexosamine biosynthetic pathway [O-linked β-N-acetylglucosamine (O-GlcNAc)], enhanced protein kinase C (PKC) signalling, oxidised small dense LDL, hyperinsulinaemia (decreased phosphatidylinositol-(3,4,5)-trisphosphate/protein kinase B pathway and increased mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase pathway), and platelet-activating factor (PAF)[25-27]. The enhanced oxidative stress is associated with endothelial dysfunction, which is characterised by increased vascular permeability and impaired vasodilation, the latter mediated by decreased NO, increased endothelin 1, and increased angiotensin II levels. Moreover, there is increased risk for leukocyte/platelet adhesion, thrombosis, and inflammation due to release of adhesion molecules including ICAM-1 and VCAM-1. The hyperleptinaemia and hypoadiponectinaemia are also associated with endothelial dysfunction and transmigration of LDL particles[28].

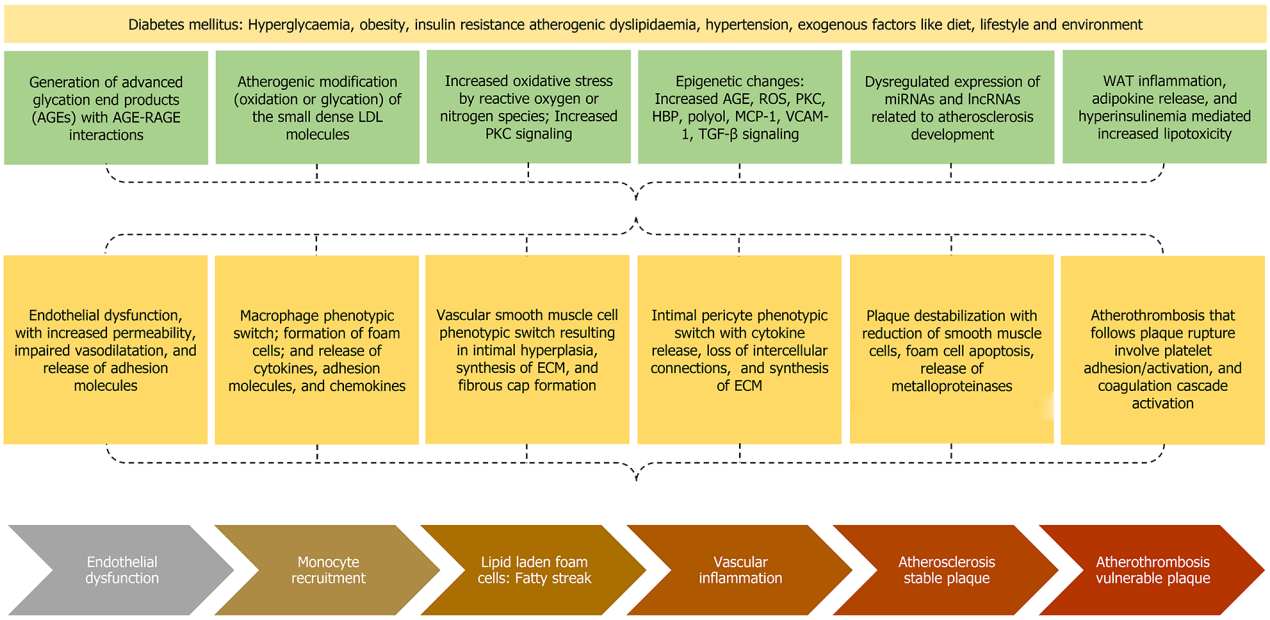

Figure 1 Pathophysiology of coronary artery disease in diabetes. AGE: Advanced glycation end products; RAGE: Receptors for AGE; LDL: Low density lipoprotein; ROS: Reactive oxygen species; PKC: Protein kinase C; HBP: Hexosamine biosynthetic pathway; MCP-1: Monocyte chemotactic pLrotein-1; VCAM-1: Vascular cell adhesion molecule 1; TGF-β: Transforming growth factor-β; ECM: Extracellular Matrix.

The enhanced PKC signalling results either from oxidative stress or the direct effect of hyperglycaemia. The enhanced PKC signalling results in increased cytokine production, increased extracellular matrix (ECM) production, and endothelial dysfunction characterised by decreased NO production, impaired vasodilation, and increased permeability[23]. Hyperglycaemia can result in heritable modifications in gene expression without changing the DNA sequences[29]. These epigenetic modifications include increased AGEs formation, increased oxidative stress, upregulation of polyol pathway, upregulation of hexosamine biosynthetic pathway, enhanced PKC signalling, enhanced TGF-β-smad-MAPK signalling, enhanced Nuclear factor-kB (NF-kB) dependent monocyte chemotactic protein-1 (MCP-1) and VCAM-1 expression. These epigenetic changes are associated with the development of endothelial dysfunction and atherosclerosis. Dysregulated expression of distinct microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are involved in various steps leading to atherosclerosis including lipid metabolism, endothelial dysfunction, vascular smooth muscle cell (VSMC) phenotypic switch, macrophage phenotypic switch, platelet reactivity/aggregation, and cardiomyocyte differentiation from stem cells as well as cardiomyocyte apoptosis[29].

The macrophage phenotypic switch from anti-inflammatory M2 to pro-inflammatory M1 phenotype results in the release of various cytokines (TNF-α, IL1-β, IL-6, and MMP), chemokines (MCP-1), and ICAM-1 release, which are mediated by increased NF-kB and activator protein-1 (AP-1) signalling. Various triggers for the macrophage switch are AGEs, polyols, O-GlcNAc, PKC, lipotoxicity, oxidative stress, mitochondrial dysfunction, and epigenetic modifications[30]. The VSMC phenotypic switch is associated with proliferation, intimal migration, and dedifferentiation into several phenotypes including synthetic, calcific, adipogenic, and macrophagic phenotypes. These changes result in intimal hyperplasia, synthesis of ECM, development of fibrous cap, and deposits of microcalcification within the intimal wall, all of which are thought to be mediated *via* TGF-β signalling[30,31].

The intimal pericyte phenotypic switch results in accumulation of lipid in pericytes through phagocytosis, secretion of pro-inflammatory cytokines, loss of intercellular connections, and increased production of ECM components[32]. Plaque destabilization, a process converting a stable plaque into a thrombosis-prone plaque, is characterised by foam cell apoptosis producing a large necrotic core, reduction of VSMCs in the fibrous cap, and release of metalloproteinase enzymes that thin down the fibrous cap resulting in the development of a vulnerable plaque[33]. Plaque rupture is followed by the development of atherothrombosis which involves platelet adhesion, platelet activation, and activation of coagulation cascade by tissue factor, plasminogen activator inhibitor-1 (PAI-1), and fibrinogen that is favoured in the presence of lowered antithrombin activity[34].

Clinicopathologic characteristics of CAD in patients with diabetes

CAD tends to be a more complex disease in patients with DM. It is characterized by diffuse, calcified, rapidly progressing disease with multivessel involvement, that often requires coronary revascularization in addition to optimal medical therapy[20]. Moreover, both culprit and non-culprit lesions in patients with DM exhibit more vulnerable features (lipid-rich core, macrophage accumulation, and thin fibrous cap), indicating pan-vascular plaque instability[35]. Diabetic patients constitute one quarter of all cases undergoing coronary revascularisation procedures such as percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). Diabetic patients with multivessel disease respond superiorly to CABG compared to PCI. However, CABG is not feasible in many diabetic patients due to the presence of complex atherosclerotic lesions and associated comorbidities. With technological advancement and newer devices like drug-eluting stents, PCI can be used in complex lesions such as those found in diabetic patients. However, the revascularisation outcomes are still worse in patients with diabetes compared to those without[36]. Among patients with CAD undergoing revascularisation with drug eluting stents, the presence of diabetes is associated with nearly double the rate of MI, definite stent thrombosis and cardiovascular mortality[37]. Moreover, patients with diabetes have 86% higher rates of in-stent restenosis[38]. Among patients admitted with acute coronary syndrome treated by CABG, the presence of diabetes increased the long-term mortality by 1.6-fold, though the 30 d mortality remained equal between those with or without diabetes[39].

Diagnosis of CAD in diabetes

In patients with CAD, presence of diabetes is associated with a twelvefold higher risk of future adverse cardiac events including mortality or non-fatal MI compared to its absence[40]. In coronary angiographic studies, the angiographical significance exhibits a poor correlation with haemodynamic (ischaemic) significance. Therefore, fractional flow reserve (FFR) is used as the gold-standard invasive technique to diagnose the coronary artery stenoses of sufficient hemodynamic severity to induce myocardial ischaemia. Myocardial perfusion imaging (MPI) with single-photon emission computed tomography is a non-invasive method to detect coronary artery stenoses of sufficient hemodynamic severity to induce myocardial ischaemia[41]. The invasive FFR or noninvasive MPI studies exhibiting an absence of ischaemia do not provide the same degree of confidence in patients with diabetes compared to patients without diabetes. Therefore, deferring revascularization based upon the absence of ischaemia (known as ischaemia-driven revascularisation strategy) does not appear to be safe in patients with diabetes as compared to those without.

There are various possible explanations for this phenomenon, including a high prevalence of microvascular dysfunction, rapid atherosclerosis progression, high atherosclerotic disease burden, and high-risk plaque composition (bigger necrotic core and larger calcium content) among the diabetic population[41]. In patients with DM, various risk factors related to atherosclerotic plaque progression include hypertension, male sex, mean plaque burden > 75% at baseline, and glycaemic variability[42]. The atherosclerotic burden can be measured invasively with the gold standard intravascular ultrasound or noninvasively with the coronary computed tomography angiography and coronary artery calcium (CAC) score. Patients with DM and metabolic syndrome have high CAC score, and in these subjects, the CAC score is a strong predictor of cardiovascular disease and mortality[43,44].

Though most patients with CAD present with angina, it may be absent in 20%-30% of patients with diabetes[45]. Baseline investigation with electrocardiogram (ECG) is of little value, but stress tests using either treadmill or dobutamine stress echocardiogram should be considered for diagnostic workup in suitable patients[46]. Similarly, myocardial perfusion scans can be used which have 88% sensitivity and 84% overall specificity[47]. In T2DM patients, numerous biomarkers could allude to an increased risk of CAD. These markers can be classified into established biomarkers related to lipid regulation (LDL, high density lipoprotein, and very low density lipoproteins) and novel biomarkers including biomarkers related to inflammation (fibrinogen or high-sensitivity C-reactive protein), lipid-associated biomarkers [Lipoprotein associated PA2 or Lipoprotein (a)] and organ-specific biomarkers (hs-troponin, Cystatin C or NTproBNP)[48]. However, no single biomarker has been found to be completely reliable. Hence, using multiple biomarkers is the way forward in reaching a timely diagnosis of CAD[49,50].

Small dense LDL particles are more atherogenic due to their specific characteristics, which include lower binding affinity to LDL receptors, higher penetration into the

subendothelial layer, longer half-life, and lower resistance to oxidative stress[49]. Glycated apolipoprotein B can be used as a surrogate marker of subclinical atherosclerosis[51]. Higher lipoprotein (a) levels are associated with poor CVD outcomes in patients with DM and is a predictor of recurrent CVD events[52]. The Triglyceride Glucose index (TyG) calculated using fasting glucose and triglyceride levels can be used as a surrogate marker of insulin resistance and subclinical atherosclerosis, to predict cardiovascular events and to detect asymptomatic coronary artery stenosis in patients with T2DM[53]. Moreover, TyG can be used as a marker to predict the progression of coronary atherosclerosis[54].

Management of CAD in diabetes

In 2007, the New England Journal of Medicine published a meta-analysis that demonstrated an increased risk of MI and other cardiovascular events with the use of antidiabetic drug rosiglitazone. Thereafter, the United States Food and Drug Administration (FDA) made it mandatory to undertake cardiovascular outcome studies for all antidiabetic medications before receiving final approval. Cardiovascular outcome trials of various antidiabetic drugs currently available are given in Table 1. The cardiovascular outcome trials have uncovered unexpected benefits of cardiovascular protection with some of the new classes of agents, such as the glucagonlike peptide-1 receptor agonists (GLP-1 RAs) and the sodium-glucose cotransporter-2 (SGLT-2) inhibitors[55].

Statin therapy is associated with reduced risk of development and progression of CAD, with a consequent reduction of cardiovascular events and mortality. In a meta-analysis of 18686 subjects, during a mean follow-up of 4.3 years, the all-cause mortality was reduced by 9% in patients with diabetes (relative risk or RR 0.91, 95%CI: 0.82-1.01; $P = 0.02$), whereas the same was reduced by 13% in those without diabetes (0.87, 95%CI: 0.82-0.92; $P < 0.0001$), for each one mmol/L reduction in LDL cholesterol[56]. The major cardiovascular adverse events were lowered by 21% in those with diabetes (0.79, 95%CI: 0.72-0.86; $P < 0.0001$) and without diabetes (0.79, 95%CI: 0.76-0.82; $P < 0.0001$). Moreover, there was a reduction in MI or coronary death (0.78, 95%CI: 0.69-0.87; $P < 0.0001$), coronary revascularisation (0.75, 95%CI: 0.64-0.88; $P < 0.0001$), and stroke (0.79, 95%CI: 0.67-0.93; $P = 0.0002$) in patients with diabetes[56].

Weight loss interventions (bariatric surgery), proprotein convertase subtilisin-kexin type 9 inhibitors, novel molecules that could block the actions of RAGE signalling, RNA therapeutics that target miRNAs and long noncoding RNAs, and drugs that target distinct components of the immune or inflammatory response hold promise as new modalities of treatment for prevention of cardiovascular disease in patients with DM[55].

DM AND CAN

Diabetic autonomic neuropathy can be classified into CAN, sudomotor neuropathy, gastrointestinal autonomic neuropathy, and urogenital autonomic neuropathy[57]. CAN is associated with impairment of autonomic control of the cardiovascular system and is a major cause of silent cardiovascular events in patients without overt cardiac disease[58]. Though the prevalence of CAN is highly variable, it is estimated to affect at least 20% of unselected patients and up to 65% of those with increasing diabetes duration and age[59]. It is increasingly observed in patients with prediabetes and metabolic syndrome, with a reported prevalence of up to 11% and 24%, respectively[60]. The sole risk factor for the development of CAN in T1DM patients is dysglycaemia, whereas the risk factors for the development of CAN in T2DM patients include dysglycaemia, dyslipidaemia, hypertension, obesity, metabolic syndrome, smoking, increasing age, microvascular complications, and low vitamin B12 or vitamin D levels[57].

Clinical features of CAN in diabetes

The autonomic nervous system involvement in CAN occurs in an ascending length-dependent manner. Therefore, the vagus nerve, which is the longest parasympathetic nerve, is involved early resulting in parasympathetic denervation and sympathetic predominance. Patients at this early stage will be asymptomatic (sub-clinical stage) and the diagnosis can only be made based on heart rate variability (HRV), baroreflex sensitivity tests or increased left ventricular torsion on cardiac imaging[61]. Towards the end stage of disease, sympathetic denervation results in unresponsiveness of heart rate and blood pressure to sleep, exercise, stress, or chemical stimulation such as

Table 1 Cardiovascular outcome trials of available antidiabetic drugs[131-150]

Drug	Trial name	Primary outcome	HR (95%CI)
Metformin[a]	UKPDS legacy data[131]	MI	0.67 (0.51-0.89)
Gliclazide MR	ADVANCE[132]	CV death, MI, stroke	0.94 (0.84-1.06)
Pioglitazone[a]	Meta-analysis[133]	CV death, MI, stroke	0.83 (0.72-0.97)
Rosiglitazone	RECORD[134]	CV event or CV death	0.99 (0.85-1.16)
Acarbose (Chinese population)	ACE[135]	CV death, MI, stroke, UA, HF hospitalisation	0.98 (0.86-1.11)
Nateglinide	NAVIGATOR[136]	CV death, MI, stroke, HF hospitalisation	0.94 (0.82-1.09)
Saxagliptin	SAVOR-TIMI[137]	CV death, MI, ischaemic stroke	1.01 (0.88-1.15)
Alogliptin	EXAMINE[138]	CV death, MI, stroke, UA, and HF	0.98 (0.86-1.12)
Sitagliptin	TECOS[139]	CV death, MI, stroke, UA hospitalisation	0.98 (0.89-1.09)
Linagliptin *vs* glimepiride	CAROLINA[140]	CV death, MI, stroke	0.98 (0.84-1.14)
Empagliflozin[a]	EMPA-REG[141]	CV death, MI, stroke	0.86 (0.74-0.99)
Canagliflozin[a]	CANVAS[142]	CV death, MI, stroke	0.86 (0.75-0.97)
Dapagliflozin	DECLARE[143]	CV death, MI, ischaemic stroke	0.93 (0.84-1.03)
Ertugliflozin	VERTIS-CV[144]	CV death, MI, stroke	0.97 (0.85-1.11)
Lixisenatide	ELIXA[145]	CV death, MI, stroke, UA	1.02 (0.89-1.17)
Liraglutide[a]	LEADER[146]	CV death, MI, stroke	0.87 (0.78-0.97)
Semaglutide[a]	SUSTAIN-6[147]	CV death, MI, stroke	0.74 (0.58-0.95)
Exenatide	EXSCEL[148]	CV death, MI, stroke	0.91 (0.83-1.0)
Dulaglutide[a]	REWIND[149]	CV death, MI, stroke	0.88 (0.79-0.99)
Albiglutide[a]	HARMONY[150]	CV death, MI, stroke	0.78 (0.68-0.90)

HR: Hazard ratio; CI: Confidence interval; CV: Cardiovascular disease; MI: Myocardial infarction; UA: Unstable angina; HF: Heart Failure.
[a]$P < 0.05$.

adenosine. At this stage, patients become symptomatic with light-headedness, weakness, palpitations, fainting and syncope on standing. Other features that can be associated with CAN include reduced exercise tolerance, silent myocardial ischaemia, intraoperative complications (hypotension, bradycardia, and the need for vasopressor support), and foot complications including foot ulcers due to associated sudomotor dysfunction, Charcot neuroarthropathy and lower limb amputations[62]. Poor response of heart rate and blood pressure during exercise in turn fail to increase cardiac output accordingly, leading to poor exercise tolerance.

The signs associated with CAN include tachycardia, orthostatic hypotension, reverse dipping, and non-dipping on ambulatory blood pressure monitoring[59]. Orthostatic hypotension indicates an advanced stage of CAN and suggests a poor prognosis with higher mortality in diabetic patients with CAN[63]. Orthostatic hypotension is mainly attributable to the damage to the efferent sympathetic vasomotor fibres, particularly in the splanchnic vasculature. Other contributing factors are reduced cardiac output, postprandial blood pooling, insulin-induced hypotension and volume depletion due to diuretics[64]. Patients can sometimes present with postural orthostatic tachycardia syndrome, which is characterised by the presence of orthostatic symptoms occurring on standing, an increase in heart rate of ≥ 30 beats/minute when moving from a recumbent to a standing position that lasts more than 30 seconds and the absence of orthostatic hypotension (more than 20 mmHg drop in systolic blood pressure)[65].

Reverse dipping (reversal of normal physiological drop in blood pressure at night) and non-dipping are commonly found in diabetic patients with CAN, due to sympathetic predominance during the night, leading to the development of nocturnal hypertension and left ventricular hypertrophy[66]. Patients with CAN are susceptible to silent myocardial ischaemia and/or infarction. There is a prolongation of subjective anginal threshold, and there is a delayed and diminished appreciation of ischaemic

pain, associated with early ECG changes prior to the onset of ischaemic symptoms[62]. Asymptomatic ischaemia can induce lethal arrhythmias, which is especially important in patients with CAN who have prolonged QT interval[67].

Pathophysiology of CAN

Pathophysiology of CAN is the same as any other form of diabetic neuropathy and is contributed by hyperglycaemia and dyslipidaemia (Figure 2). Hyperglycaemia is the main driver of diabetic neuropathy in T1DM, whereas dyslipidaemia is the main driver in T2DM patients[68]. Diabetes is associated with high substrate load of glucose and free fatty acids. In the presence of hyperglycaemia, glucose enters the Schwann cells through glucose transporter 3 (GLUT3). Excess glucose undergoes glycolysis, and pyruvate exceeds the capacity of tricarboxylic acid (TCA) cycle, resulting in a shift to anaerobic metabolism and accumulation of lactate. Lactate is shuttled from Schwann cells into axons, resulting in mitochondrial dysfunction and axonal degeneration[69].

Moreover, hyperglycaemia results in excessive activation of the electron transport chain, leading to mitochondrial dysfunction, ROS generation, oxidative stress, DNA damage, and activation of poly adenosine diphosphate ribose polymerase. The latter, in turn, inhibits glyceraldehyde-3-phosphate dehydrogenase resulting in accumulation of glycolytic metabolites, with upregulation of polyol, hexosamine, and diacylglycerol (DAG) and PKC pathways, as well as generation of AGEs[70-76]. The AGE-RAGE interactions, oxidative stress, endoplasmic reticulum (ER) stress and upregulated non-glycolytic pathways result in endothelial dysfunction characterized by impaired vasodilation mediated by decreased NO bioavailability, increased endothelin-1, increased PAI-1 and aberrant angiogenesis. Angiogenesis is increased in diabetic nephropathy and retinopathy, whereas it is decreased in diabetic neuropathy[70]. Microvascular damage decreases neuronal blood flow resulting in demyelination, axonal loss, decreased myelinated fibre density, and reduced nerve conduction velocity[76].

Common mechanisms for the excessive ROS generation in patients with hyperglycaemia include excessive activation of the electron transport chain, AGE-RAGE interaction, pro-inflammatory cytokines, upregulated non-glycolytic pathways, and high protein folding load. Crosstalk exists between oxidative stress and ER stress[70]. AGE accumulation and hexosamine/polyol pathway upregulation are associated with a rise in misfolded or unfolded proteins and, therefore, ER stress. Oxidative stress increases the misfolding of proteins with worsening ER stress. Similarly, misfolded proteins result in adenosine triphosphate depletion, thereby increasing oxidative stress[70,75].

A high substrate load of long chain saturated fatty acids including palmitate and stearate is associated with increased β oxidation to form acetyl CoA. When the capacity of the TCA cycle is exceeded, toxic acylcarnitine accumulates inside Schwann cells, which is then shuttled into axons, resulting in mitochondrial dysfunction and axonal degeneration[68]. In addition, oxidation of cholesterol into oxysterols in neuronal cells results in neuronal injury and apoptosis[77,78]. Furthermore, altered sphingolipid metabolism with the generation of neurotoxic deoxysphingolipids can be another mechanism for nerve damage in T2DM patients with diabetic neuropathy[79,80].

Lifestyle factors like high-calorie diets are associated with alterations in gut microbiota and the emergence of metabolic endotoxemia. The metabolic endotoxaemia activates the Toll-like receptors 4 to create a sympatho-vagal imbalance characterised by an increased proinflammatory sympathetic outflow and a decreased anti-inflammatory parasympathetic outflow, culminating in perivascular adipose tissue inflammation, systemic inflammation, neuronal inflammation and CAN[58]. Obstructive sleep apnoea is associated with chronic intermittent hypoxia, increased oxidative stress, and microvascular dysfunction[59]. Other factors implicated in the pathogenesis of CAN include genetic and epigenetic changes, autoimmune autonomic ganglionopathy, and low C-peptide levels indicating a poor pancreatic β cell reserve[62].

Diagnosis of CAN

The CAN Subcommittee of Toronto Consensus Panel on Diabetic Neuropathy recommends that T2DM patients should be screened for CAN at the time of diagnosis, and T1DM patients should be screened for CAN within 5 years of their diagnosis. The committee also recommends that the screening should be done as a part of perioperative risk assessment in patients with CAD[81]. American Diabetes Association (ADA) recommends screening for CAN in patients with microvascular complications, neuropathic complications, and hypoglycaemia unawareness[82]. CAN should be suspected in diabetic and prediabetic patients when they present with resting tachycardia, postural tachycardia, reduced HRV, bradycardia, impaired exercise

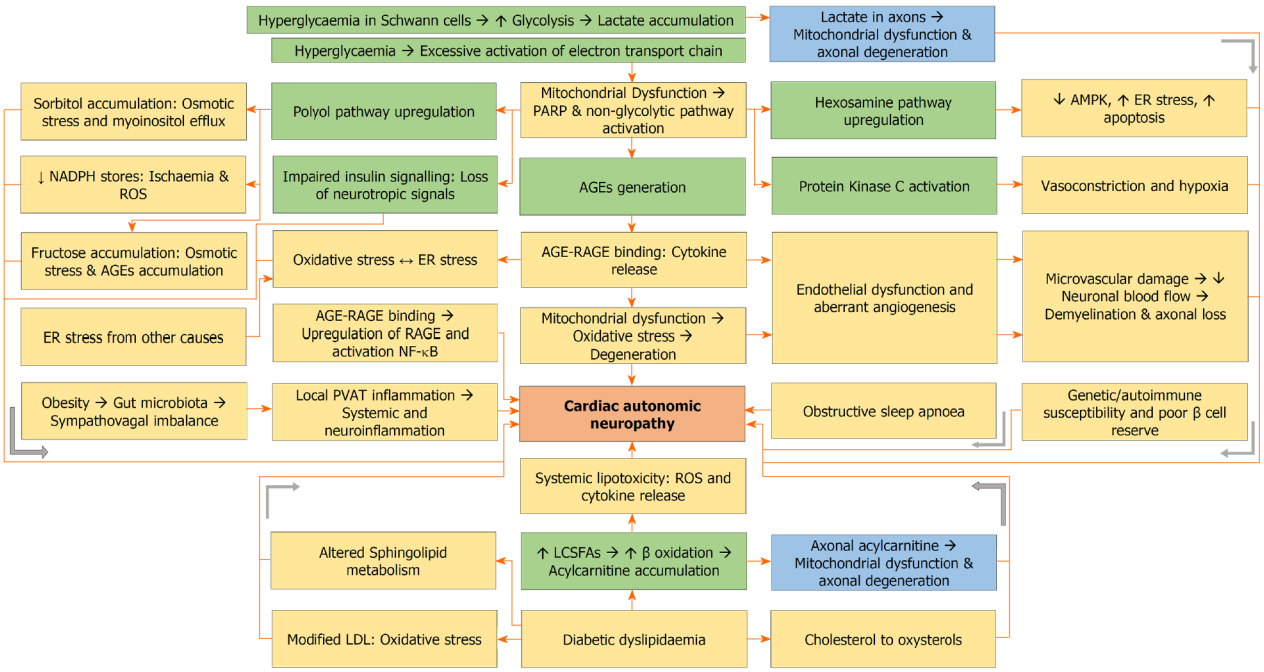

Figure 2 Pathophysiology of cardiac autonomic neuropathy in diabetes. PARP: Polyadenosine-diphosphate-ribose polymerase; AMPK: Adenosine monophosphate-activated protein kinase; ER: Endoplasmic reticulum; ROS: Reactive oxygen species; NADPH: Nicotinamide adenine dinucleotide phosphate; AGE: Advanced glycation end products; RAGE: Receptors for AGE; LDL: Low density lipoprotein; LCSFA: Long chain saturated fatty acid.

tolerance, orthostatic hypotension, supine hypertension, or intra-operative or post-operative cardiovascular instability[57].

The gold standard for diagnosis of CAN is Cardiovascular Autonomic Reflex Testing (CARTs), which consists of tests of parasympathetic function, including HRV to deep breathing, heart rate response to standing, and heart rate response to Valsalva manoeuvre; tests of sympathetic adrenergic function including beat-to-beat blood pressure response to Valsalva manoeuvre, and the systolic and diastolic blood pressure changes in response to tilt table or active standing; and tests of sympathetic cholinergic function including quantitative sudomotor axon reflex test, thermoregulatory sweat testing, and sympathetic skin response. Based on the results of CARTs, the diagnosis can be early/possible CAN (1 abnormal cardiovagal test), definite/confirmed CAN (2 abnormal cardiovagal tests), and severe/advanced CAN (2 abnormal cardiovagal tests with orthostatic hypotension)[81].

Morbidity and mortality associated CAN

Even from the subclinical stage, CAN is independently associated with an increased risk for cardiac arrhythmias, silent myocardial ischaemia, major adverse cardiovascular events, myocardial dysfunction, sudden cardiac death (from either silent myocardial ischaemia or QT interval prolongation), all-cause mortality, and cardiovascular mortality[60]. In patients with diabetes, the 5-year mortality from the time of diagnosis of CAN is 16%-50%[83]. The risk for cardiovascular disease is increased proportionately to the stage of CAN, the faster the progression, the greater the cardiovascular disease risk[84].

Patients with CAN are at high risk of developing HF with preserved ejection fraction (HFpEF), an entity that has a significantly high mortality risk. Subclinical CAN with parasympathetic denervation and sympathetic predominance results in high myocardial catecholamine levels, high myocardial oxygen demand, left ventricular hypertrophy, left ventricular remodelling, myocardial apoptosis and fibrosis, all of which manifest as HFpEF[85]. Moreover, CAN may contribute to the progression of atherosclerosis through many mechanisms including a rise in heart rate and blood pressure, trophic changes in the arterial wall, arterial stiffness, and a pro-inflammatory state associated with autonomic neuropathy[86].

Management of CAN

As it is associated with adverse cardiovascular consequences, interventions to prevent or reverse CAN should be implemented, as parasympathetic denervation may be

reversible if diagnosed soon after onset[87]. ADA suggests optimising glycaemic control in T1DM patients, multifactorial interventions targeting glycaemia and other cardiovascular risk factors in T2DM patients, and lifestyle modifications in patients with prediabetes with or without metabolic syndrome for the prevention of CAN[82]. Though some drugs developed on the basis of pathophysiological mechanisms have shown promise in the preclinical trials, but all failed in obtaining approval. Therefore, there are currently no FDA-approved disease-modifying drugs for the management of CAN. Symptomatic patients with orthostatic hypotension can be treated with either fludrocortisone or midodrine, and those with postprandial hypotension can be treated with octreotide, using the same principles in the management of neurogenic orthostatic hypotension[88].

DM AND HF

Elderly subjects with diabetes have a high incidence of HF compared to those without diabetes (39% *vs* 23%), and those without HF at baseline have a relative risk of 1.3 for developing HF after 43 months of observation[89]. Diabetes increases the prevalence of HF by threefold in patients less than 75 years of age and by twofold in patients more than 75 years of age[90]. The prevalence of HF in subjects with diabetes is 12%-57%, whereas the prevalence of diabetes in subjects with HF is 4.3%-28%[90]. Moreover, HF including HFpEF and HF with reduced ejection fraction (HFrEF) often coexist with T2DM in nearly 30%-40% of cases, with the HFpEF being commoner than HFrEF[90,91]. Both T1DM and T2DM are associated with increased risk for HF in women compared to men, with T1DM associated with 47% greater risk and T2DM associate with 9% greater risk[92]. Hyperglycaemia in patients with DM promotes the development of HF. Each 1% increment in HbA1c is associated with a 30% increase in HF in patients with T1DM and 8% increase in HF in patients with T2DM[93,94]. HF in subjects with diabetes is associated with increased rates of hospitalisation, cardiovascular mortality, and all-cause mortality, with the greatest risk for patients with HFpEF, compared to patients with HFrEF[95,96].

Pathophysiology HF in DM

HF in patients with DM could arise from a combination of DCM, ischaemic cardiomyopathy, CAN, and hypertensive cardiomyopathy. The pathogenic mechanisms that are known to cause DCM and CAN, namely, hyperglycaemia, insulin resistance, and hyperinsulinaemia are also risk factors for the development of ischaemic heart disease and hypertension among patients with DM[25].

DCM

DCM is defined as a condition of cardiac dysfunction encompassing myocardial metabolic, structural, and functional changes in the absence of other cardiac risk factors, such as CAD, hypertensive heart disease, and significant valvular disease, in individuals with T1DM or T2DM[97,98]. In the early stages, DCM remains asymptomatic and the only sign is an increase in left ventricular mass (hypertrophy), which is independent of hypertension and body weight, and results from dysfunctional cardiac remodelling. In the next stage, myocardial fibrosis develops, leading to diastolic dysfunction, which is the most common manifestation of DCM. The diastolic dysfunction can be asymptomatic during the early stages or can present with HFpEF. A small number of people with DCM progress to develop overt systolic dysfunction presenting as HFrEF[99].

Left ventricular diastolic dysfunction develops early during diabetes and is detected in up to 75% of T2DM patients[90]. A recent study estimated the prevalence of left ventricular diastolic dysfunction among diabetic patients in the hospital population and the general population and observed a prevalence of 48% in the former and 35% in the latter group[100]. The same team estimated the prevalence of left ventricular systolic dysfunction in the hospital population and general population separately as 18% and 2%, respectively[101]. The degree of glucose dysregulation was proportional to the severity of diastolic dysfunction, risk of incident HF and cardiovascular mortality in patients with T2DM. Nearly 50% of HF patients with T2DM have HFpEF, especially the older, female, hypertensive patients. Accurate diagnosis of HFpEF is often difficult as symptoms are often mild, and hence misdiagnosis is common. Though CAD is the major cause of HFrEF in patients with T2DM, other causes like DCM should be considered in the differential diagnosis[90].

Pathophysiology of DCM

Under normal conditions, the cardiac energy demand is met by fatty acid oxidation, with a small contribution from glucose. However, under stressful situations, the cardiomyocytes rely on increased contributions from glucose[102]. The ability to use a variety of fuels to generate energy is known as metabolic substrate flexibility[99]. In adult cardiomyocytes, glucose enters the cell *via* GLUT4, whereas fatty acid enters *via* fatty acid translocase (FAT or CD36)[103]. Insulin resistance is associated with decreased GLUT4-mediated glucose uptake and increased CD36-mediated fatty acid uptake into the cardiomyocytes, with sole reliance on fatty acid for fuel. The glucose metabolism that accompanies hyperinsulinaemia and chronic hyperglycaemia is associated with an increased generation of ROS and oxidative stress. The oxidative stress will divert glucose metabolism from its usual glycolytic pathway to alternative pathways including the polyol pathway, resulting in the generation of AGEs, or the hexosamine biosynthetic pathway (HBP) resulting in the generation of O-GlcNAc[104].

The ROS and AGE (following interaction with receptors for AGE or RAGE) trigger activation of NF-kB, thereby inducing inflammation mediated by TNF-α, IL-6, IL-8, and MCP-1 and myocardial fibrosis mediated by TGF-β and MMP[103,105]. The AGE-RAGE interactions result in crosslink between collagen and elastin as part of ECM remodelling, which in turn leads to increased myocardial stiffness and impaired relaxation[104]. Moreover, ROS triggers ER stress in cardiomyocytes resulting in their apoptosis, and impaired myocardial calcium handling leading to cardiac dysfunction[104]. The chronic activation of HBP with the generation of O-GlcNAc also results in impaired myocardial calcium handling[106]. Following apoptosis, the viable cardiomyocytes undergo compensatory hypertrophy, which is associated with a shift in myosin heavy chain (MHC) from α-MHC to β-MHC and upregulation of atrial natriuretic peptide and brain natriuretic peptide (BNP)[107].

In patients with insulin resistance, increased fatty acid uptake is associated with increased oxidative and non-oxidative fatty acid metabolism, with the latter resulting in the generation of toxic intermediates including ceramide and DAG[108]. Once the mitochondrial oxidative capacity is exceeded, this results in mitochondrial dysfunction, generation of ROS, oxidative stress, ER stress, generation of inflammatory cytokines, and lipotoxicity mediated apoptosis (lipoapoptosis)[104]. Hyperglycaemia is associated with inappropriate activation of the renin-angiotensin-aldosterone system (RAAS) with a rise in angiotensin II and aldosterone and increased expression of mineralocorticoid receptors, increasing the oxidative stress, cardiac fibroblast proliferation, and cardiomyocyte hypertrophy[109]. Coronary endothelial dysfunction, in the form of impaired NO and endothelium-derived hyperpolarising factor mediated vasodilation, leads to microvascular dysfunction, myocardial ischaemia and contractile dysfunction.

Exosomes are extracellular vesicles that are released by cardiovascular system-related cells, including cardiomyocytes, endothelial cells, fibroblasts, smooth muscle cells, platelets, leukocytes, monocytes, and macrophages[110]. These exosomes are mediators of intercellular communication, and contain a variety of biological components like miRNAs, proteins, and lipids. Exosomal dysregulation is a mechanism for the pathogenesis of DCM. The exosomes released by cardiomyocytes to endothelial cells inhibit proliferation, migration, and tube formation of endothelial cells, leading to microvascular dysfunction. The exosomes released by endothelial cells to cardiomyocytes cause inhibition of cardiomyocyte autophagy, and promotion of cardiomyocyte apoptosis. Moreover, the exosomes released by fibroblasts to cardiomyocytes serve as a mediator of cardiomyocyte hypertrophy[110].

The key components in the pathogenesis are myocardial fibrosis, left ventricular remodelling, and cardiac dysfunction[111]. Activation of proinflammatory cytokines, production of ROS, dysfunction of mitochondria and ER in cardiac tissue are the key operating mechanisms that contribute towards cardiac remodelling, fibrosis, and diastolic dysfunction. These are triggered due to the combined effects of enhanced deposition of AGEs and lipotoxic metabolites, activation of RAAS, altered calcium homeostasis and abnormal insulin signalling pathways through activation of the mammalian target of rapamycin (mTOR)-S6 kinase 1 pathway leading to abnormal intracellular glucose transport in cardiomyocytes[112]. Figure 3 illustrates the complex pathophysiological mechanisms involved in the development of DCM.

Diagnosis of DCM

Currently, there are no specific clinical features, structural/functional changes, or biomarkers for the diagnosis of DCM. In the very early stages, there are only substructural changes in the cardiomyocytes, and the diagnosis is only possible

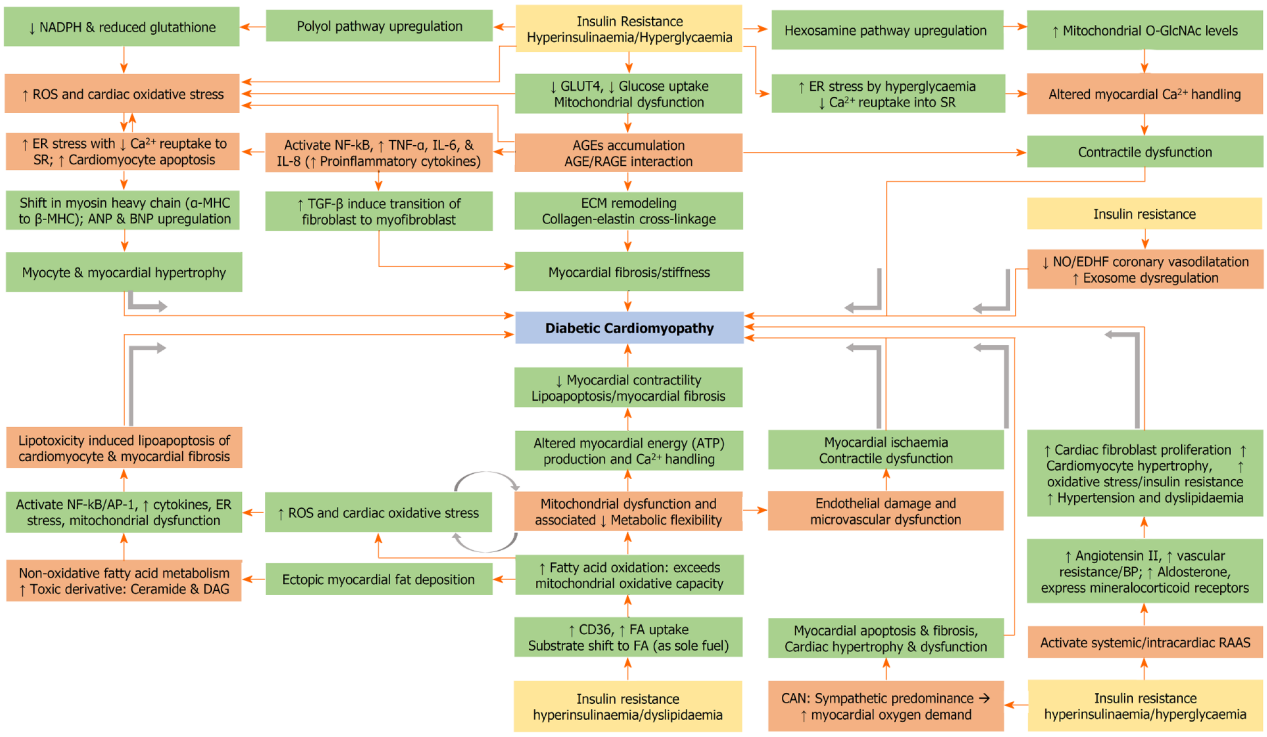

Figure 3 Pathophysiology of diabetic cardiomyopathy. NADPH: Nicotinamide adenine dinucleotide phosphate; ROS: Reactive oxygen species; GLUT: Glucose transporter; ER: Endoplasmic reticulum; SR: Sarcoplasmic reticulum; NF-kB: Nuclear factor-kB; TNF-α: Tumour necrosis factor-α; IL-6: Interleukin-6; IL-8: Interleukin-8; AGE: Advanced glycation end products; RAGE: Receptors for AGE; MHC: Myosin heavy chain; ANP: Atrial natriuretic peptide; BNP: Brain natriuretic peptide; TGF-β: Transforming growth factor-β; ECM: Extracellular matrix; NO: Nitric oxide; EDHF: Endothelium-derived hyperpolarising factor; ATP: Adenosine triphosphate; AP-1: Activator protein-1; BP: Blood pressure; DAG: Diacyl glycerol; FA: Fatty acid; CAN: Cardiac autonomic neuropathy; RAAS: Renin-angiotensin-aldosterone system.

through very sensitive methods including longitudinal myocardial strain alterations (a measure of tissue deformation), strain rate and myocardial tissue velocity, which are indicators of early adverse left ventricular remodelling[113]. In the middle stages of DCM, when cardiomyocyte hypertrophy and fibrosis develop with associated structural changes like left ventricular hypertrophy and increased muscle mass, non-invasive imaging technologies including echocardiography and gadolinium-enhanced cardiac magnetic resonance imaging (MRI) will be able to detect diastolic and/or systolic dysfunction. In advanced stages of DCM, there will be worsening of fibrosis and development of microvascular changes, and this stage will be often accompanied by overt HF, ischaemic heart disease and hypertension[113].

Transthoracic echocardiography can be used for evaluation of structural changes (2D echo for left ventricular mass), and functional changes (trans-mitral doppler for diastolic dysfunction and tissue doppler imaging for diastolic and systolic dysfunction) in patients with DCM[114], even though the diastolic dysfunction observed in patients with T2DM is worsened when hypertension and obesity coexist[115]. The ratio between early passive trans-mitral inflow velocity (E) and velocity at the medial mitral annulus (e') can be used as a measure of left ventricular filling pressure[116]. Abnormal E/e' is correlated with the development of HF and increased mortality, independent of other risk factors such as hypertension and CAD[117]. Gadolinium-enhanced cardiac MRI is much more accurate than echocardiography for the evaluation of structural changes (myocardial fibrosis, steatosis, and left ventricular mass), functional changes (for diastolic and systolic function: late gadolinium enhancement), and metabolic changes (Magnetic Resonance spectroscopy for myocardial triglyceride content and high-energy phosphate metabolism)[118]. Positron emission tomography, which could diagnose DCM at much earlier stages, can be used to assess myocardial metabolic abnormalities. However, this is still a research tool as it is costly, time-consuming, and needs expertise for accurate interpretation of the results[103].

Although diastolic dysfunction diagnosed invasively through cardiac catheterisation (left ventricular end-diastolic pressure > 16 mmHg or mean pulmonary capillary wedge pressure > 12 mmHg) is the most definitive evidence of diastolic HF, it is rarely necessary for the diagnosis of DCM, due to the availability of various highly

sensitive and specific noninvasive tests[119]. However, coronary angiography has an added benefit of detecting CAD, including microvascular CAD. Various biomarkers are undergoing investigation as markers of structural changes (matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase, for myocardial fibrosis) and functional changes (mi-RNA for contractile function, procollagen 3 N-terminal peptide and troponin for LV dysfunction, and BNP for LV diastolic and systolic function)[114].

Management of DCM

Glycaemic control alone is insufficient to prevent the development of DCM, indicating the need for targeted therapeutic strategies. Some of the newer antidiabetic drugs such as the GLP-1 RAs and the SGLT-2 inhibitors have exhibited direct protective effects on the myocardial tissue[120]. Therapeutic approaches including RNA-based therapy (a form of gene therapy), drugs targeting the mitochondrial oxidative stress, and metabolic modulator drugs including trimetazidine, ranolazine, perhexiline, alpha lipoic acid, resveratrol, luteolin, riboflavin, sodium ferulate, cyclovirobuxine, and epigallocatechin-3-gallate are currently being investigated for the prevention and treatment of DCM[121-123].

EMERGING THERAPEUTIC AGENTS FOR CARDIOVASCULAR DISEASE PREVENTION

Precision medicine is a rapidly advancing field of medicine. It is based on the principle of identification of the underlying molecular pathophysiological mechanisms of disease and the design of specific therapeutic interventions against these mechanisms[124]. The messenger RNA is the coding RNA, whereas the various regulatory RNAs which are not translated into proteins are known as non-coding RNAs. The different regulatory non-coding RNAs include small interfering RNAs (siRNAs), miRNAs, lncRNAs, and circular RNAs. The siRNAs and miRNAs are 20-22 nucleotides in length, and these are involved in the post-transcriptional regulation of gene expression. The lncRNAs that are > 200 nucleotides in length are involved in the regulation of transcription, splicing and in the regulation of siRNAs and miRNAs[125].

RNA sequencing technologies have identified that various non-coding RNAs have a role in the pathogenesis of cardiovascular disease. These non-coding RNAs can be used as treatment targets. RNA therapeutics, a form of gene therapy, has received widespread application to target the RNA molecules and regulate gene expression and protein production. The non-coding RNAs that are associated with cardiovascular disease can be packaged into viral vectors including adenovirus, lentivirus, or adeno-associated virus, and can be delivered into the target cells to mediate therapeutic benefits by regulating the expression of the target gene[126]. Inhibition of endogenous miRNA and lncRNA can be achieved by administering antisense oligonucleotides that are complementary to their sequences[125]. Smaller non-coding RNAs like miRNAs and siRNAs can also be delivered into the cells using various chemical vehicles like lipid mixtures (lipofection), lipid nanoparticles, or dendrimers; or using various biological vehicles including minicells or exosomes[127].

RNA therapeutics delivered using viral vectors have certain advantages such as the ease of generating vectors, highly efficient transduction, and long-term stable gene expression, whereas the non-viral delivery methods like oligonucleotide-based therapies have advantages like ease of dosage control, low immunogenicity and zero risk of genomic integration. The exosome-mediated and nanoparticle-mediated delivery methods might further improve the efficiency and accuracy of RNA-based therapeutics[126]. However, gene expression might still be uncontrollable with the abovementioned methods. Recently, a delivery system based on probiotic bacteria has been developed, which is more efficient in reaching a high level of gene expression and is more controllable as host bacteria can be manipulated[128]. In summary, manipulation of non-coding RNAs by silencing the hazardous non-coding RNAs or administering beneficial on-coding RNAs could reduce cardiovascular disease in patients with DM[129]. Moreover, RNA binding proteins are important in post-transcriptional gene expression. Many RNA binding proteins and RNA binding protein-regulated RNA networks are disrupted in patients with DM and diabetic complications. Thus, RNA binding proteins provide another therapeutic option for the prevention of cardiovascular disease in patients with DM[130].

CONCLUSION

Diabetic heart disease is a conglomeration of CAD, CAN, and DCM as shown in Figure 4. CAD associated with diabetes tends to be a more complex disease and is characterised by diffuse, calcified, rapidly progressing disease, with more vulnerable features and multivessel involvement that often requires coronary revascularization in addition to optimal medical therapy. However, revascularisation outcomes are still worse in patients with diabetes compared to those without diabetes. CAN should be suspected in diabetic and prediabetic patients when they present with resting tachycardia, postural tachycardia, reduced HRV, bradycardia, impaired exercise tolerance, orthostatic hypotension, supine hypertension, or intra-operative or post-operative cardiovascular instability. CAN is independently associated with an increased risk of cardiac arrhythmias, silent myocardial ischaemia, major adverse cardiovascular events, myocardial dysfunction, sudden cardiac death, all-cause mortality, and cardiovascular mortality. DCM is characterised by cardiac dysfunction encompassing metabolic, structural, and functional myocardial changes developing in individuals with DM, in the absence of CAD, hypertensive heart disease, and significant valvular disease. Diastolic dysfunction may be asymptomatic during the early stages or can present with HFpEF. A small number of people progress to develop overt systolic dysfunction presenting as HFrEF. Glycaemic control alone is insufficient to prevent the development of diabetic heart disease. Control of traditional risk factors such as dylipidaemia, hypertension, and smoking are important, along with the appropriate use of various preventive medications such as statins, antiplatelet agents and RAAS modifiers. Some newer antidiabetic drugs such as the GLP-1 RAs and the SGLT-2 inhibitors have exhibited a direct cardioprotective effect. Many drugs based on the pathophysiological mechanisms including RNA therapeutics are under development.

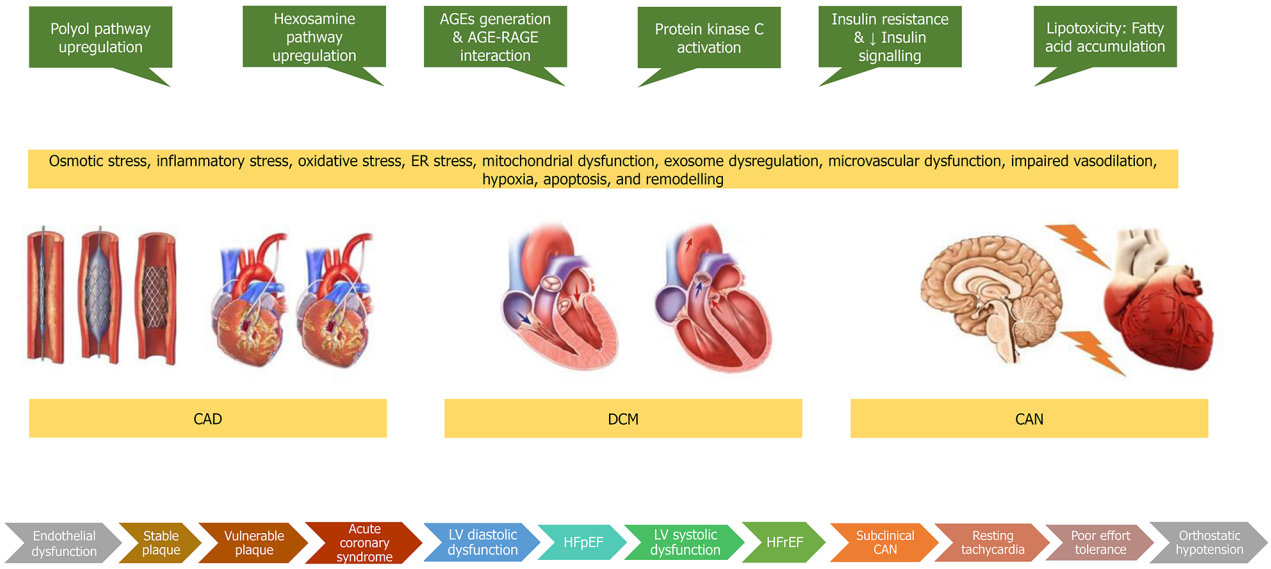

Figure 4 Pathophysiology and clinical presentation of diabetic heart disease. AGE: Advanced glycation end products; RAGE: Receptors for AGE; HFpEF: Heart failure with preserved ejection fraction; HFrEF: Heart failure with reduced ejection fraction; CAN: Cardiac autonomic neuropathy; CAD: Coronary artery disease; DCM: Diabetic cardiomyopathy.

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MINIREVIEWS

Alphabet strategy for diabetes care: A checklist approach in the time of COVID-19 and beyond

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Abstract

Chronic disease management requires achievement of critical individualised targets to mitigate again long-term morbidity and premature mortality associated with diabetes mellitus. The responsibility for this lies with both the patient and health care professionals. Care plans have been introduced in many healthcare settings to provide a patient-centred approach that is both evidence-based to deliver positive clinical outcomes and allow individualised care. The Alphabet strategy (AS) for diabetes is based around such a care plan and has been evidenced to deliver high clinical standards in both well-resourced and under-resourced settings. Additional patient educational resources include special care plans for those people with diabetes undertaking fasting during Ramadan, Preconception Care, Prevention and Remission of Diabetes. The Strategy and Care Plan has facilitated evidence-based, cost-efficient multifactorial intervention with an improvement in the National Diabetes Audit targets for blood pressure, cholesterol levels and glycated haemoglobin. Many of these attainments were of the standard seen in intensively treated cohorts of key randomized controlled trials in diabetes care such as the Steno-2 and United Kingdom Prospective Diabetes Study. This is despite working in a relatively under-resourced service within the United Kingdom National Health Service. The AS for diabetes care is a useful tool to consider for planning care, education of people with diabetes and healthcare professional. During the time of the coronavirus disease 2019 pandemic the risk factors for the increased mortality observed have to be addressed aggressively. The AS has the potential to help with this aspiration.

Key Words: Alphabet strategy; COVID-19; Care planning; Chronic disease; Diabetes care; Multifactorial interventions; Patient care

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Core Tip: The alphabet strategy for diabetes care is a very useful tool for the effective long-term management of patients with diabetes. It is based on; advice on lifestyle, blood pressure targets, cholesterol targets and chronic kidney disease prevention, diabetes control, eyecare, footcare, guardian drugs where indicated. This is achieved through an approach that is similar for both health care professionals (HCP) and patients. This is achieved through care planning, HCP guidelines and specialised care plans (for Ramadan, for example). It is an evidence based, patient centred, health care professional assisted, low-cost approach for treatment of diabetes and prevention of long-term diabetes complications.

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INTRODUCTION

One of the major global public health burdens of this current era, with over 450 million people affected globally, is type 2 diabetes mellitus[1]. Advances in acute care have led to increased life expectancy for patients with diabetes through excellent emergency management of conditions such as acute coronary syndrome, stroke and sepsis. The challenge now is to effectively manage their chronic disease to reduce the other complications of diabetes[1]. This has become especially important as the coronavirus disease 2019 (COVID-19) pandemic continues to spread globally with diabetes mellitus being identified as a significant risk factor for mortality (increased risk: × 2.86 for type 1 diabetes, × 1.80 for type 2 diabetes)[2]. Additionally, poor glycaemic control, obesity, Black and South Asian ethnicities, and chronic kidney disease (CKD) were identified as significant risks for increased mortality in patients with diabetes and COVID-19[3].

A multifactorial approach for diabetes management to reduce complications is encouraged in the United Kingdom through the National Institute for Health and Care Excellence (NICE) guidelines, Scottish Intercollegiate Guidelines Network guidelines and the National Diabetes Audit (NDA). A recent report published by NDA showed that meeting all three treatment targets (glycated haemoglobin, blood pressure, statin prescription) was only achieved in 19.6% for type 1 diabetes and 40.5% for type 2 diabetes patients (In England)[4]. Our institutional average of meeting these targets was 46.5%. It also observed that the percentage of patients receiving NICE recommended care processes has not shown significant improvement from 2012 to 2019.

CONCEPT OF CARE PLANNING

"Care planning" involves the process by which both health care workers and patients have detailed discussions on the condition that the patient has. An individualized plan of management is then agreed based on the patient's personal values and aspirations for their life. The written document after this process of planning is the "Care plan"[5]. The action plan and interventions required to manage an acute condition are very different than what is required to manage a chronic condition. At the same time, all chronic conditions, may if they differ in the involvement of organs of human body, have a common set of problems which need to be addressed by patient, their families and health carers. This concept is motivated by the physical, psychological and social needs which arise due to the chronic conditions of the patients. Care planning may save the enormous expenditure on chronic diseases which usually runs in billions of pounds[6]. It is important to remember that in an average year of 8766 h, a patient with diabetes will only spend approximately 45 to 90 min with a health care professionals (HCP). This could be two to four appointments of 10-30 min each, rest of the 8764.5 h in the year the patient has to self-manage (personal data based on clinic survey).

Care planning is not only being implemented across different countries but also across different specialties. Though care planning can be distinguished in terms for the conditions or for the patients, in usual practice it is most often for a condition-specific

basis[5]. The care planning content can reflect the perspective of health professional or patient, the extent of the plan to which the behaviour change is intended, and the spread of the plan (*i.e.* involving only doctor-patient or involving doctor-patient-multidisciplinary teams/social teams)[7]. Care planning also involves behaviour change and subsequent other techniques to sustain those behaviour changes[8]. Care plans are considered to be one of the best tools for standardisation of care processes[5]. Prompts on electronic records such as those used in general practice in United Kingdom also serve this purpose.

Evidence for multi-factorial intervention

Rawshani *et al*[9] investigated the increased risk of cardiovascular events and mortality in type 2 diabetes patients in comparison to general population[9]. This study was conducted on 271174 patients with type 2 diabetes from the Swedish National Diabetes Register who were matched with 1355870 controls without diabetes. The strongest predictors for combined cardiovascular outcomes and death were- current smoking, blood pressure (BP) ≥ 140/80 mmHg, low-density lipoprotein ≥ 2.5 mmol/L, albuminuria (micro or macro), HbA_1c ≥ 53 mmol/mol. Each of these five factors increased the risk of acute myocardial infarction, stroke, heart failure and mortality in patients with diabetes. The more the number of these risk factors present in a patient with diabetes, the higher was the risk observed for both unfavourable cardiovascular outcomes and mortality in comparison to the control group. This study reflects that multifactorial intervention in patients with diabetes may strongly impact in not only decreasing the adverse cardiovascular outcomes, but also reducing the mortality in diabetic patients. In the cohort < 55 years of age, the results showed that the increased risk in those with none of these five risk factors *vs* those with all 5 of the risk factors was: (1) excess mortality × 4.99; (2) excess myocardial infarction × 11.35; (3) excess stroke × 7.69; and (4) excess heart failure × 7.69.

Similar results were obtained from the follow-up Steno-2 study which recruited 160 patients with type 2 diabetes with microalbuminuria[10,11]. There were two randomized groups- one group receiving conventional multifactorial treatment and other group receiving intensified target driven therapy (targets included HbA_1c, fasting serum total cholesterol and triglyceride levels, systolic and diastolic BP). At the start of the initial trial both groups were similar at baseline but developed significant differences by the end, showing that the intensive therapy was better than conventional therapy in attaining the set targets. In the follow up trial, both groups had received the intensive therapy and the gap of differences was observed to be narrowed by the end of the follow-up trial. The conclusions from the entire trial suggested that there is an absolute risk reduction (ARR) of 20% for death from any cause and an ARR of 29% for cardiovascular events in the intensive therapy group. Moreover, progression of diabetic complications was significantly reduced in the intensive therapy group.

The checklist approach for patient management has been rewarding in other specialties as well. Haynes *et al*[12] used a two-step surgical checklist in eight hospitals eight cities in different geographical parts of the world[12]. Their intervention with the checklist showed marked improvements in outcomes as reduction of major surgical complications and rate of death.

The alphabet strategy for diabetes

Our innovation in diabetes care was the "Alphabet strategy" (AS), which aimed for "simple things to be done right all the time"[13]. It is a mnemonic based checklist incorporating the core diabetes care components[14]; and includes the following (Figure 1): (1) advice on lifestyle: Diet, weight, and physical activity optimisation, not smoking, safe alcohol use, appropriate infection control [and very specifically *vs* severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)], nationally advised vaccinations (such as against influenza, SARS-CoV-2, pneumococcus); (2) BP: < 140/80 mmHg, ≤ 130/80 mmHg if kidneys or eyes affected or any cardiovascular disease; (3) cholesterol and CKD prevention: Total cholesterol ≤ 4 mmol/L, screening for and treating microalbuminuria; (4) diabetes control: HbA_1c ≤ 58 mmol/mol or an individualised target, avoiding hypoglycaemia and hyperglycaemia interventions (for example diabetes ketoacidosis). Individualised glucose monitoring plans; (5) eye examination: check yearly at least, with referral if indicated; (6) foot care: Daily inspection and examination by patient and yearly examination by HCP; and (7) guardian drugs: Aspirin, clopidogrel and angiotensin converting enzyme inhibitors or angiotensin receptor blockers as indicated for primary and secondary preventions of cardiovascular and renal diseases.

The first version of the AS was published in 2002[14]. Since then the clinical guidance and Care Plans have been updated yearly. The strategy has proven to be adaptable

Figure 1 Alphabet Strategy poster for clinics.

and it has been relatively straight forward to adapt it to even the COVID-19 pandemic. Because of the curtailment in services during the COVID-19 pandemic we have incorporated an H for Healthcare Professional advice as follows.

Healthcare Professional advice: Especially for problems such as recurrent hypoglycaemia, recurrent diabetic keto-acidosis, difficulty accessing care (including affordability), Ramadan advice, pre-conception advice, driving and occupational advice, remission of diabetes.

The clinical impact of AS was assessed by two audits done in outpatient settings of a district level hospital in United Kingdom. The first audit involved 420 patients with diabetes who were followed over 5 years. The second audit, performed 2 years after the completion of the first audit, involved 1071 patients with diabetes. Comparison of the outcomes of the two audits showed improvement in all AS components (Table 1). An audit conducted in a low-income country (India), with 100 patients with diabetes in an outpatient clinic, also demonstrated the effectiveness of AS (Table 2).

The potential of AS was globally assessed by a survey carried out in the Global AS Implementation Audit. It involved 4537 patients from 52 centres across 32 countries. The data showed that the strategy was highly acceptable to both patients and their healthcare professionals in high, middle and lower income countries.

This new care plan was developed with the National Health Service (NHS) England and NHS Improvement West Midlands our Region Diabetes Expert Advisory Group, Right Care, and the Pharmacy Local Professional Network Chair (Figure 2).

The AS core training materials (slides, documents and videos) have been created to facilitate and disseminate the programme. The training pack distributed to individual patient groups such as Diabetes United Kingdom (Figure 3) consists of:

Patient education posters: Educate the patients about the AS overall. There is a similar poster for diabetes care for specifically during Ramadan developed with the South Asian Health Foundation (Figure 4).

Patient care plans: Empowering to know their NDA target and improve self-efficacy in attaining these. It also guides the important steps for pregnant patients to reduce complications (maternal and foetal). The main components of the care plan are as follows: (1) background information: Personal targets based on NDA, Diabetes United Kingdom 15 Healthcare Essentials, Key contacts; (2) patient's agenda: Tick section for patients to indicate points for discussion, Questions to reflect on health status and specific goals; (3) patient's personal agenda: Aspects of care to be documented such as BP, Cholesterol, Creatinine and Urine Albumin Creatinine Ratio, HbA_1c, eye screening, feet examination, guardian drugs; (4) drugs and glucose monitoring: The HCP should advice on the dosing, frequency and side effects of any new drugs started for the patient. Advice for the patient on monitoring of blood glucose with an appropriate Glucose Testing Meter including frequency of monitoring. Patient should have Community Pharmacist review also where available; and (5) contact details: There is a list of the important contact details for the key specialists/organizations related to diabetic patient management.

One page guideline: Summary of one-page current NICE guidelines in relation to diabetes care (Figure 1) to achieve higher rates of NDA attainment and facilitate multifactorial interventions. This includes sections on Diabetes Prevention and Diabetes Remission.

Referral guidelines: Diabetes Expert Advisory group advice has been incorporated on who to refer and who not to refer (Figure 5). Clear guidelines are given by Red Amber Green on whom to refer to the specialist diabetes team from primary care and secondary care. Such advice, if implemented, has the potential to reduce hospital admissions and reduce costly complications such as amputation and end-stage renal disease.

Glucose monitoring advice and choice of meter: This has the potential to save £1.74 million in our region if all Clinical Commissioning Groups (CCGs) merely adopted current practice similar to the best performing CCG. Enhancement on top of this can save £6.3 million *per* year (NHS England and NHS Improvement-West Midlands). Our region has already saved £ 960000 in 12 mo.

Drug optimisation advice: Data from our region shows that adopting best practice across the region would save £1.65 million on one class of diabetes drugs alone, example dipeptidyl peptidase-4 inhibitors (NHS England and NHS Improvement-West Midlands modelling data).

Prevention of diabetes advice: There is 86% chance of remission in selected newly diagnosed patients. Data for this comes from the DiRECT Study[15].

Achieving diabetes care excellence through primary care team programme: This is a low cost (£25) basic e-learning platform that covers all the main aspects of diabetes care and care planning using the AS. It is hosted by Coventry University Health Sciences Faculty. It is also incorporated into Sound Doctor's Diabetes Education Program for patients, which is Quality Institute for Self-Management Education and Training approved.

Outcomes from the AS

Improvement in process measures: Implementation of the AS for Diabetes Care resulted in a significant ($P < 0.05$) improvement including lipid measurement, BP,

Table 1 Comparison of achievement of alphabet strategy components between practices of evidence-based medicine audits[10]

Alphabet strategy		Baseline audit, *n* = 420	Follow-up audit, *n* = 1071	*P* value
A	Smoking status (%)	15.5	14.7	0.83
B	Blood pressure (mmHg)	141/77	136/76	0.007
C	Total cholesterol (mmol/L)	4.9	4.5	< 0.001
	LDL cholesterol (mmol/L)	2.5	2.4	< 0.001
	Creatinine (mmol/L)	109	105	0.036
D	HbA1c (%) (mmol/mol)	8.3 (67.2)	7.9 (62.8)	0.09
E	Eye examination (%)	95.5	97.1	0.72
F	Foot examination (%)	83.5	97.3	< 0.001
G	Aspirin (%)	83.5	88.0	0.20
	ACEI/ARB (%)	73.0	74.4	0.75
	Lipid lowering (%)	55.0	73.4	< 0.001

Follow-up audit was done 2 years later. ACEI: Angiotensin converting enzyme inhibitors; ARB: Angiotensin receptor blockers; LDL: Low-density lipoprotein.

Table 2 Change in care process performance following implementation of the alphabet strategy in a low-resource diabetes clinic[10]

		Pre implementation (%)	Post implementation (%)	*P* value
A	Body mass index	99	99	NS
	Smoking status	99	99	NS
	Smoking cessation	100	100	NS
B	Blood pressure	99	99	NS
C	Total cholesterol	60	99	< 0.001
	Lipid profile	10	64	< 0.001
	Creatinine	5	49	< 0.001
	Proteinuria	48	93	< 0.001
D	Fasting and postprandial glucose	41	97	< 0.001
E	Eye examination	98	100	NS
F	Feet examination	95	100	NS
G	Aspirin therapy	6	71	< 0.001
	ACEI/ARB therapy	7	57	< 0.001
	Statin therapy	5	38	< 0.001
	All three	2	20	< 0.001

NS: Not significant; ACEI: Angiotensin converting enzyme inhibitors; ARB: Angiotensin receptor blockers.

HbA_1c, eye and foot examinations. Using the parameters from NDA, this strategy showed 100% performance of seven of the NICE recommended processes[16]. Our unit scored above average in six out of the seven categories for target care process achievement.

Improvement in outcome measures: The improvement rates (Table 1) were comparable to standards achieved in clinical trial setting specifically researching intensive treatment strategies like Steno-2 and The United Kingdom Prospective Diabetes Study.

Patient and health care professional satisfaction: An audit conducted in 27 countries

Diabetes NICE Clinical Guidelines 2021: Locally Adapted Guidelines
Diabetes Care: the Alphabet Strategy Approach

Advice on Lifestyle:

General

- **Smoking cessation, physical activity, diet, weight control (5-10% loss/year if overweight). Details below**
- **Structured education:** especially self-management, beliefs, knowledge, skills, driving, occupation
- **Regular follow-up with Care Planning. Annual Review is essential.** 20% with early severe complications will be persistent Diabetes Clinic non-attenders. Ramadan advice. Advise Diabetes UK membership.

Diabetes Prevention Lifestyle (PH 38) and for diagnosed Diabetes

- **Physical Activity:** choose activities that are enjoyed and fit into daily lives. At least 150 minutes (2½ hours) of moderate intensity activity in bouts of 10 minutes or more, eg: 30 mins./ 5 days a week. Or 75 mins. vigorous intensity activity across the week or combinations of moderate and vigorous intensity activity. Also resistance physical activity to improve muscle strength at least two days a week. Minimise being sedentary (sitting)
- **Weight management:** encourage overweight and obese people to gradually reduce calorie intake. Explain 5–10% weight loss in 1 year is realistic initial target. Use evidence-based behaviour-change techniques. Motivate and support to achieve and maintain – a healthy BMI. General population, 18.5-24.9 kg/m^2, South Asian or Chinese descent, 18.5 and 22.9 kg/m^2. Orlistat an option (as below).
- **Dietary advice:** Advise the right amount of calories for the level of activity (daily usually: men 2,500 cals., women 2,000 cals). Most adult/some children have too many calories from carbs. Ensure protein intake adequate. **Satiety: protein > fat > carbs.** Ensure ≥ 3 fruit & veg/day. Cut down on saturated fat (eg butter, cheese, cakes, sausages) to < 30g men, 20g women. Cut down on sugars. Salt < 6g/day. Carbs: more complex. Don't confuse thirst with hunger. Smaller regular meals. Don't skip breakfast.
- **Metformin :** HbA1c rising despite participation intensive lifestyle program **or** unable to participate. Particularly if BMI > 35. Explain long-term lifestyle change can be more effective than drugs in preventing or delaying T2DM. Continue lifestyle advice. Check renal function before Rx, then x2 yearly or more. Start low dose (eg 500 mg od), increase to 1500–2000 mg daily. If intolerant, consider metformin MR. Prescribe for 6–12 months. Monitor HbA1c or fasting plasma glucose at 3-month intervals and stop the drug if no effect.
- **Orlistat:** Use clinical judgement on whether to offer orlistat if BMI ≥ 28.0 kg/m^2 for obesity. Discuss benefits & side effects. Advise low-fat diet (<30% daily energy as fat, over 3 main meals). Review use after 12 weeks. If weight loss not at least 5%, ?stop Rx. Use orlistat for > 12 months, only after discussing benefits & side-effects

Diabetes Remission Protocol (DIRECT Study):

- **If diabetes duration < 6 yrs:** 830 cal diet for around 12 weeks (calories from: protein 26%, fat 13%, carbs 61%). Then 400 cal. meals introduced. Vitamins and minerals replete. Off all anti-diabetic and anti-hypertensive Rx. Optimal Physical Activity advised (ideally 15000 steps per day). Relapse with weight gain treated.
- **86% chance of remission at 1 year if ≥15kg weight loss. 57% remission if 10-15kg weight loss.**

Blood pressure: *National Diabetes Audit target < 140/80, ≤ 130/80 if kidney, eye or any CVD*

- **Step 1.** Age < 55 yrs: **A** (ACEI or ARB). ≥ 55 yrs or African-Caribbean **C** (Ca^{2+} blocker) **or D** (indapamide)
- **Step 2. A + D or A +C** : **Step 3. A + C + D**
- **Step 4. Add** K^+ sparing diuretic (e.g. spironolactone) or α-blocker (doxazosin) or β-blocker (eg bisoprolol)

Cholesterol: *NDA < 5mmol/l, NICE > 40% reduction in non-HDL Chol. Secondary Prevention*

Primary Prevention: Type 1 DM:

- **Atorvastatin 20mg od if >40years or duration > 10 years or established nephropathy or other CVD risk factors**

Primary Prevention: Type 2 DM: Atorvastatin 20mg od if ≥ 10% 10 year CVD risk on **QRISK 2**

Secondary Prevention (all): Atorvastatin 80mg od. CVD (MI, angina, stroke, TIA, PVD). Initiate lower dose Atorvastatin if older, low muscle mass, impaired renal function or patient preference

CKD Patients:

- **Atorvastatin 20mg. If > 40% reduction in non-HDL cholesterol not achieved, increase dose. Agree use of high-dose statin with renal specialist if eGFR <**

Other Rx: Ezetimibe 10mg and/or Fenofibrate 160mg/200mg may be useful in statin intolerance to reach targets. Hydrophilic Pravastatin and Rosuvastatin less side-effects (simvastatin side-effect profile increased with amlodipine, diltiazem, verapamil, > 250ml of grapefruit juice daily). Bempedoic acid and PSCK9-i: specialist advice.

CKD Prevention: Micro Alb: ACEI, or ARB. Ramipril 10mg daily daily data shows stroke reduction, MACE reduction and mortality reduction by 24%. **Proteinuria**: 20-28% reduction death/ESRD (losartan 100mg od), also SGLT2i.

Diabetes control: Individually-agreed targets. *NDA HbA1c ≤ 58mmol/mol (≤7.5%)* ***individualized***

- **Type 2 Initial Rx:** Lifestyle (optimal diet, optimal weight, physical activity), Metformin 500mg bd, 850mg bd, 1000mg bd (usual doses). Contraindicated if creat. > 150 umol/l or eGFR < 30 ml/min. Consider B12 check
- **Type 2 First Intensification: Individualise to pt: If non-obese** SU eg: gliclazide start low dose eg 40mg od then titrate eg 80 mg bd, 160 mg bd max- note hypo risk. **If obese:** SGLT-2i (weight loss). If CKD adjust dose if needed. DDP-4i (weight neutral), Pioglitazone or GLP-1RA sc also options. **Consider Insulin** if ketones high, losing weight, marked symptoms & glucose > 15 mmol/l or very high HbA1c (>86 mmol/mol)
- **Type 2 Second Intensification: Individualise to pt:** Use appropriate 3rd line agent from above choices
- **Type 2 Third Intensification: Individualise to pt:** Appropriate agent from above ? insulin ? GLP-agonist sc
  - **Insulin regimes:** NPH, glargine, levemir, degludec, toujeo overnight, biphasic bd, basal bolus regimes.
  - **GLP- agonists: once-weekly Semaglutide or Dulaglutide. Avoid Semaglutide in those with any grade above background retinopathy if on insulin and poor glycaemic control.** Consider instead of insulin or TZD especially if BMI ≥ 30 if problems with ↑ weight, occupation issues, insulin unacceptable or weight loss would benefit co-morbidities
- **New Type 2 Guidelines: EASD/ADA guidance:** If clinical CVD: SGLT-2i or GLP-1RA with proven CV benefit is recommended. If CKD, Clinical heart failure or High CVD risk CVD, a SGLT-2i inhibitor with proven benefit is recommended. GLP-1RA are generally recommended as first injectable Rx.
- **Type 1:** Insulin essential to life. Use suitable regime usual basal bolus, premix bd in some patients. Classic symptoms may not be present eg: ketones high, losing weight, marked symptoms with hyperglycaemia > 15 mmol/l. Aim no/minimal hypoglycaemia. DKA avoidance. Safe Driving advice. Consider flash monitoring, CGMS, Pump therapies as per guidelines. **NB: Metformin is insulin sparing in obese Type 1 . Dapagliflozin licensed in Type 1 DM for improving glycaemic control. Watch out for rare euglycaemic DKA – sick day rules esssential.**

Eye screening: Screening for and effective management of Diabetic Retinopathy.

- BP and Glycaemic Control essential.
- Screen annually using a digital retinal camera. Aspirin/ACE-I/ARB in most patients with retinopathy. Consider fenofibrate.- some evidence of reduced need for laser Rx is diagnosed retinopathy. Several national unit use it for maculopathy (FIELD Study reduction in retinal laser and other outcomes by 34%)

Feet screening: Foot care advice and Annual review essential by GP, Practice Nurse or podiatrist.

- All risk factors to be controlled aggressively.
- Inspection, pedal pulses, 10g MF testing. If neuropathic or ischaemic, foot-care advice and regular podiatry review essential to prevent ulceration/amputation. Ulcers: refer urgently to MDT Foot At Risk Team.
- In the FIELD Study there was a 36% reduction in amputation using fenofibrate 160mg od. ? consider in individual cases with previous amputation?

Guardian drugs:

- **Aspirin 75mg od when BP <150 systolic**: in any atheromatous CVD. Clopidogrel 75 mg if further atheroma events on aspirin or aspirin intolerance.
- **ACEI reduce complications.** ***Ramipril 10mg od consider for most diabetes pts*** *(Best Evidence in T2DM)*
- **ARB:** Microalbuminuria (Best evidence: Irbesartan 300mg od) also if ACE not tolerated. Proteinuria to retard progression to death and ESRD (Best evidence: losartan 100mg od)

NHS England (West Midlands) Diabetes Expert Advisory Group c/o vinod.patel@warwick.ac.uk

NB: No statins, No ACE-Is, No ARBs in Pre-conception or Pregnant, 15% Foetal malformation. Pre-conception Care Essential (Folate 5mg od, Vit D 400 IU) Aim HbA1c% ≤ 7.5% = 58mmol/mol

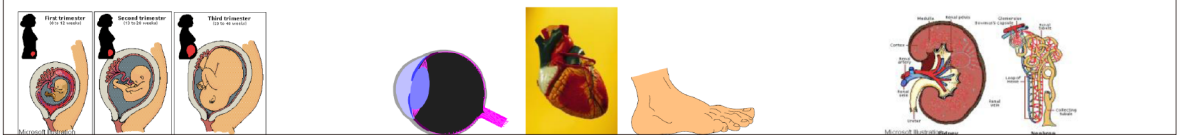


Figure 2 New care plan was developed with the National Health Service England and National Health Service improvement west midlands our region diabetes expert advisory group, right care, and the pharmacy local professional network.

(44 Diabetes service units) showed that 91% of respondent felt that the strategy would have a positive influence on diabetes care and that it would be practical to implement, even in a non-high income country (Table 2)[13].

A

- **Your diabetes** should not prevent you living a normal, healthy life. This Care Plan using the Alphabet Approach can help you.
- **Personalised Targets:** You should work towards achieving these for BP, Cholesterol and Diabetes control. National targets have been advised by Diabetes UK.
- **Diabetes UK advises 15 Healthcare Essential Standards** These can be discussed at your consultation.
  - **Specialist team to provide long term care**
  - **Safe driving and work-related advice**
  - **High-quality care if admitted to hospital**
  - **Specialist care if planning to have a baby**
  - **Opportunity to discuss any sexual problems**
  - **Access to psychological support if needed**
- **Diabetes Complications:** the risk of these can be reduced by achieving your targets. All Diabetes Complications can be reduced: **heart disease, strokes, amputation, blindness, kidney disease and hospital admissions for complications.** And a good outcome for mother and baby in pregnancy
- **Coronavirus Pandemic-Watch for symptoms:** Persistent cough, fever, change in taste or smell. Good Infection Control: esp. handwashing, distancing, face coverings,

Key Contact	How can they help	Contact Details
GP Practice Nurse	Management and Co-ordination of Care	
Pharmacist	Help with medicines	
Podiatrist	Foot care	
Dentist	Oral care	
Dietitian	Diet	
Diabetes UK	Support / Info	
Eye Screening	Annual screening	
Hospital Team	Specialist Care	

B **My current health and wellbeing**

Things I would like to discuss or have help with – please tick

Smoking	Weight	Diet
Physical Activity	Blood Pressure	Cholesterol control
Diabetes Control	Eye Care	Foot Care
Driving	Travel	Sexual Health
Pregnancy		Medications
Mental Health ? depression		What to do when ill?
Other:		

Any questions I have?

What is good or has improved about my health?

Concerns I have about my current health and wellbeing

MY GOALS: To improve my health & wellbeing I want to be able to?

To achieve them I will need to do the following?

How important are they to me?

Not important 1 2 3 4 5 6 7 8 9 10 *Important*

How long will this take?

C Date:	Your target	Result 1	Result 2	National Targets
Advice on Lifestyle: •Weight and Body Mass Index:	Kg BMI	1	1	≤25
•Stop smoking: if you smoke		2	2	Non smoker
•Diet and Physical activity		3	3	Within 12 mths diagnosis
Blood Pressure: • **Yearly check:** High BP can cause heart disease, stroke, eye and kidney disease	Reading	4	4	140/80 or less
Cholesterol and CKD Prevention • **High cholesterol** can cause heart disease, stroke and poor circulation the legs with risk of amputation	Cholesterol	5	5	Less than 5 mmol/l
• **CKD: Chronic Kidney Disease** Prevention -Yearly kidney tests (Creatinine and UACR)	Creatinine UACR	6	6	Kidney tests yearly
Diabetes Control: • **HbA1c test:** measures the amount of glucose sticking to your blood in the last 2 months	HbA1C	7	7	HbA1c 58 mmol/mol or less (7.5%)
• **Hypo avoidance:** essential to avoid low glucose levels of less than 4	Avoid Hypos			
• **Driving:** Remember to check before driving: glucose 5 or more to drive				
Eyes: • It is important that your eyes are examined yearly. Treatment may be needed to stop blindness	Date last Check	8	8	Annual check
Footcare: • Examine your feet daily: check for heat (infection), ulcers, numbness, circulation. Yearly HCP examination.	Date last Check	9	9	Daily and annual check
Guardian Drugs / Flu jab • Take your medications as advised. Many are essential to avoid heart and kidney disease.		10	10	? taken regularly
Overall Comment:				

D **Main Diabetes medication:**	**What is it for**	**Additional information**
Include dose	*lower blood glucose*	*How to take, side effects*
Insulin Treatment	**Usual dose**	**Additional information**
name		
Blood glucose testing	Yes ◯	No ◯
Type of meter		
How often should you test		

Your Notes and Advice from Pharmacist, GP, Practice Nurse, Hospital
New medication started _____ Please see pharmacist

Figure 3 The alphabet strategy care plan. A: Introduction and contacts; B: Personal concerns and goals; C: National diabetes audit data and individualised target; D: Drugs and pharmacist reviews.

Treatment Changes during Ramadan

- As you know some treatments will need adjusting, for example, some drugs need changing as you cannot drink fluids as normal.
- We would advise you to change your treatment as below
- **Please go back to your normal times and doses after Ramadan**

Current Treatment	Ramadan
	Sehri (morning)
	Iftari (evening)

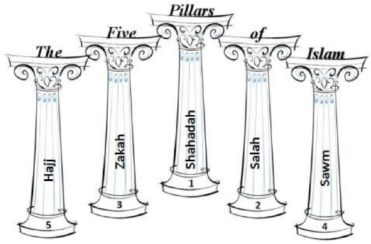
For further details contact

Diabetes Nurses: 024-76865210
Multi-Lingual Co-Worker: 024-76865595

Parveen Deen and Amitha Gopinath 2012

Ramadan and Diabetes

Fasting safely during the Holy month of Ramadan



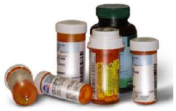
General Advice

- The Diabetes Care Team would like to help you Fast safely during Ramadan. We provide Ramadan diabetes advice in the local community and also at the hospital.
- Over-eating during Ramadan and Eid can increase your blood sugars and make you put on weight.
- Fasting allows you to abstain from smoking; Ramadan is a good time to stop smoking!
- Eat 5 portions of fruit and vegetables a day
- Ramadan is a good time to make small lifestyle changes. These changes will help you to have good control of your diabetes and reduce chance of a heart attack or stroke

Diet

- When you open your Fast limit the amount of sweet foods such as dates, milkshakes, jelabi and burfi.
- At **Sehri** and **Iftari** time eat more starchy foods, such as basmati rice, chapatti, brown bread and cereals.
- Eat more fruit, vegetables, dhal and low fat yoghurts.
- All drinks should be sugar-free, avoid adding sugar to tea and coffee. Limit the amount of salt you add to food.
- To avoid dehydration make sure you drink plenty of water before starting the Fast.
- When you break your Fast, try not to have too many fried foods such as samosas, paratha and pakoras!

Medication



- During Ramadan it is very important to keep taking your regular tablets. Some tablets will need adjusting.
- Your tablets will keep your blood glucose in control and keep you feeling well.
- If you decide to Fast and you are on insulin, you will need to be very careful, your insulin dose will need to change. Do not stop your insulin.
- **For further advice contact the diabetes team at the hospital or your own GP.**

Diabetes Control

- Check your blood glucose regularly, it should be between 4 – 7
- When your blood glucose drops below 4, you may be at risk of having a hypo. You may feel weakness, sweating, trembling, tingling in the lips and fingers and slurred speech. If this happens then you must take 2-3 glucose tablets followed by a snack.

Figure 4 Ramadan advice leaflets. This includes a table to state what change in medication may be needed during Ramadan.

CONCLUSION

In United Kingdom, care planning for all patients with chronic diseases has been proposed as an agreed action plan which is best reflected by the slogan–"no decisions about me without me"[17]. The 15 diabetes healthcare essentials introduced for the diabetes patients not only help in better management outcomes, but also help prevent serious future complications due to the disease[18]. One of the parts of the Year of Care programme for diabetes tested the "house of care" concept, which involves care and support planning at its core surrounded and supported by all the teams, tools and management plans[19]. The AS helps deliver these aspirations. The strategy is also compatible with helping implement other key national guidelines and recent changes in clinical practice[15,20-22].

AS for diabetes care is intended to improve the confidence of the person with diabetes to self-manage their condition with the aspirations and the constraints of the life they lead. We are confident that it has the great potential to reduce the morbidity and mortality due to diabetes in these most difficult of times internationally due to the COVID-19 pandemic.

All our resources are available electronically, gratis, on request. These resources are available to adapt to local clinical practice as HCP and patients see fit.

Diabetes Care Referral Criteria: COVID-19 Times

In all cases referral depends on expertise of Primary Care. In many cases, discussion will ensue with a secondary care colleague or the Community Diabetes Specialist Nurse. Format is similar to that adopted by the "Think Glucose" Campaign and the Portsmouth "Super 6" Service Model*

Primary care	In-patient care
Early Referral	**Early Referral**
(1) Inpatient diabetes* • To optimise control and safe/early discharge **(2) Foot diabetes (predefined criteria)*** • Foot Ulceration, Charcot, infection **(3) Type 1 DM, all adolescents*** • All new Type 1 Diabetes patients **(4) Insulin Pump services*** • Insulin Pump Care • New Therapies eg GLP-injectables + insulin **(5) Low eGFR/renal dialysis*** • Creatinine > 150 umol/l or CKD 3 • Proteinuria: UACR ≥ 30mg/mmol • Optimise risk factors then renal referral **(6) Antenatal diabetes*** • Any diabetes patient or Gestational DM • Pre-conception Care: asap much neglected **Other Possible Criteria:** • All patients pre Surgery with HbA1c% > 8.5% (72mmol/mol) • Individualised "Poorly controlled" : ○ HbA1c% > 9% (75 mmol/mol) ○ BP > 140/90 ○ T: Chol > 5 mmol/l or LDL > 3) • DM Acute CHD or Stroke (last 3 months) • Severe hypoglycaemia (episode requiring 3rd party assistance or HCP help) • Retinopathy requiring laser Rx or grade ≥3	• Hyperglycaemia: glucose > 12 on treatment, in pregnancy if glucose > 5.5 pre-meals and >7.7 after meals • DKA/Hyperglycaemic Hyperosmolar state • Severe hypoglycaemia • Admission for urgent/ major elective surgery • Acute coronary syndrome or Sepsis or Severe Vomiting or Impaired consciousness • Unable to self manage • Previous diabetes problem as inpatient • IV insulin infusion glucose outside limits • IV insulin for over 48 hrs • Parenteral or enteral nutrition • Foot ulceration • Newly diagnosed type 1 or type 2 diabetes • Pancreatitis in DM pt • Patient request • Gestational Diabetes (or pre-existing DM) **Gestational diabetes (GDM)** is detected by OGTT, usually at 24-28 wks. If previous GDM, OGTT carried out at 16-18 wks, followed by repeat OGTT at 28 wks if first test normal. GDM is any one of these values on OGTT or fasting: • Fasting or base-line: ≥ 5.1 mmol/l • 1 hour value: ≥ 10 mmol/l • 2 hour value: ≥ 8.5 mmol/l
Referral May Be Required	**Referral May Be Required**
• **Diabetes Care Education:** Desmond, GERTIE (Type 1 Education Programme) • **Neuropathy:** GI tract, hypotension, ED • **Diabetic "Arthritis"** eg Carpal Tunnel Syn. • **Isolated nerve palsy:** 3rd Nerve, foot drop • **PCOS** with or without Diabetes • **Obesity management:** DM with BMI > 35 • **Secondary DM:** eg steroid use, acromegaly, psychoses Rx, pancreatitis • **Low level of concordance with care** • **Pre- Ramadan advice**	• IV insulin infusion with good glucose control • Nil By Mouth more than 24hrs post-surgery • Significant educational need • Persistent hyperglycaemia • Possible Type 2 diabetes diagnosis • Stress hyperglycaemia • Poor wound healing • Steroid therapy • Pancreatitis • Discharge planning: if change in treatment needs facilitating
Referral Not Normally Required	**Referral Not Normally Required**
• Stable Diabetes care: consider Tele-health consultation • Impaired Glucose Tolerance, Impaired Fasting Glucose • New Diagnosis of type 2 Diabetes	Minor, self-treated hypoglycaemia Transient hyperglycaemia Basic educational need or routine dietetic advice Well controlled diabetes Good self-management skills, Routine care

Figure 5 Diabetes care referral criteria.

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MINIREVIEWS

Obesity, metabolic health and omics: Current status and future directions

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Abstract

The growing obesity epidemic is becoming a major public health concern, and the associated costs represent a considerable burden on societies. Among the most common complications of severe obesity are the development of hypertension, dyslipidemia, type 2 diabetes, cardiovascular disease, and various types of cancer. Interestingly, some obese individuals have a favorable metabolic profile and appear to be somehow protected from the detrimental effects of excessive adipose tissue accumulation. These individuals remain normoglycemic, insulin sensitive, and hypotensive with proper blood lipid levels, despite their high body mass index and/or waist circumference. Multiple independent observations have led to the concept of the metabolically healthy obese (MHO) phenotype, yet no consensus has been reached to date regarding a universal definition or the main mechanism behind this phenomenon. Recent technological advances and the use of high-throughput analysis techniques have revolutionized different areas of biomedical research. A multi-omics approach, which is used to investigate changes at different molecular levels in an organism or tissue, may provide valuable insights into the interplay between the molecules or pathways and the roles of different factors involved in the mechanisms underlying metabolic health deterioration. The aim of this review is to present the current status regarding the use of omics technologies to investigate the MHO phenotype, as well as the results of targeted analyses conducted in MHO individuals.

Key Words: Metabolically healthy obesity; Cardiovascular diseases; Genomics; Transcriptome profiling; Proteomics; Metabolomics

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Core Tip: Multiple independent observations have led to the concept of the metabolically healthy obese (MHO) phenotype, in which individuals, despite a high body mass index, remain normoglycemic, insulin sensitive, and hypotensive with proper blood lipid levels. Even though this issue is of great interest to the scientific community, no consensus has been reached to date regarding the main mechanism behind this phenomenon. The aim of this review is to present the current status regarding the use of omics technologies to investigate the MHO phenotype at different molecular levels, as well as the results of targeted analyses conducted in MHO individuals.

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INTRODUCTION

Over the past several decades, the incidence of obesity has tripled worldwide[1]. The high amounts of excess or abnormal adipose tissue, together with all the related comorbidities, are imposing a considerable burden on societies, and obesity has become a major public health concern. Among the most common complications of severe obesity are the development of hypertension, dyslipidemia, type 2 diabetes, cardiovascular disease (CVD), and various types of cancer[2]. There are, however, some obese individuals who exhibit a favorable metabolic profile. These individuals remain normoglycemic, insulin sensitive (IS), and hypotensive with proper blood lipid levels, despite their high body mass index (BMI) and/or waist circumference (WC). Based on multiple independent observations, the concept of the metabolically healthy obese (MHO) phenotype[3,4] has been proposed, yet no consensus has been reached to date regarding a uniform definition or the main mechanism behind this phenomenon.

Definition and prevalence

More than 30 sets of criteria for the MHO phenotype have been used in different clinical studies over the years, and most have included parameters such as BMI, blood pressure, fasting plasma glucose, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), WC, and homeostasis model assessment of insulin resistance[5]. The inconsistencies between studies are mostly related to different cutoff values for the parameters or the number of criteria that have to be met to define the MHO phenotype[6,7]. Furthermore, some researchers have applied metabolic syndrome (MetS) definitions, while others have defined metabolic health based on insulin sensitivity[8] or liver fat[9]. The variabilities in the criteria used in studies represent a significant limitation, complicating the interpretation and comparison of research results. This issue may also, in part, explain the discrepancies in the reported prevalence of the MHO phenotype, which has differed between studies depending on the type of population studied and definition used. For example, Liu *et al*[10] reported a prevalence that ranged from 4.2% to 13.6%, whereas that reported by Rey-López *et al*[11] ranged from 6% to 75%. A recent meta-analysis of data from 12 cohort and 7 intervention studies found that almost one-third of obese individuals were metabolically healthy[12]. In general, the prevalence of the MHO phenotype has been found to be higher in women and younger study participants[11,13]. Metabolically healthy individuals have also been observed to be less sedentary and more physically active than their unhealthy counterparts[14].

MHO phenotype and associated risks

The lack of clearly defined diagnosis criteria for the MHO phenotype has also led to inconsistent results among studies evaluating the association between the metabolic health status and the risk of CVD, type 2 diabetes development, and mortality. Some epidemiological studies with short-term follow-ups have reported that MHO individuals have a similar risk of incident CVD as metabolically healthy normal weight (MHNW) individuals. On the other hand, more recent studies with long follow-ups have found that MHO individuals have a higher risk of CVD and

cardiovascular mortality than healthy non-obese subjects[15]. A meta-analysis by Eckel *et al*[16] revealed that study participants with the MHO phenotype had a higher risk of developing CVD than MHNW individuals and a lower risk than metabolically unhealthy normal weight subjects, regardless of the MHO definition. Similar results were obtained by Bell *et al*[17], who investigated the incidence of type 2 diabetes among MHO individuals and found that their risk of developing diabetes mellitus was approximately half that of metabolically unhealthy obese (MUO) subjects; however, the risk was significantly higher in MHO individuals than in MHNW individuals. The most recent meta-analysis, which included 23 studies, found an increased risk of CVD in the MHO group compared with healthy lean subjects[18]. Interestingly, in this meta-analysis, the risk of CVD in MHO individuals was similar even when different numbers of risk factors were used to define good metabolic health and remained high even when none of the metabolic abnormalities were present. The MHO group was found to have a significantly increased risk of all-cause mortality, but not cardiovascular mortality, when compared to the MHNW group[18].

Mechanisms underlying the MHO phenotype

Although MHO individuals have a favorable metabolic profile, it remains unclear whether this protection from the detrimental consequences of obesity is permanent or temporary. A recent meta-analysis including 5914 MHO individuals revealed that half of them experienced a deterioration of their metabolic health over time[12]. The Multi-Ethnic Study of Atherosclerosis, which was based on data from 6809 individuals, found that the MHO phenotype was not significantly associated with the incidence of CVD; however, half of the population in the study developed MetS during the follow-up period. The conversion from the MHO to MUO phenotype was found to result in a substantially increased CVD risk. Interestingly, study participants with a stable MHO phenotype were found to have a comparable risk as MHNW subjects[19]. Therefore, maintaining a healthy metabolic status appears to be a valid approach for preventing cardiometabolic diseases.

Despite the great interest of the research community, little is known about the exact mechanism responsible for a favorable metabolic profile in the presence of obesity. Several factors and phenotypic traits have been described, including normal adipose tissue function, low liver fat content, low visceral and ectopic fat volume, preserved insulin sensitivity, and low levels of inflammation[20]. Several studies have also suggested that genetic predisposition[21], circulating micro-RNAs (miRNAs)[22], or gut microbiota[23] may play a role (Figure 1).

Aim of the review

Given all these discrepancies and the rather unclear understanding of the underlying mechanisms, this review article aims to provide an overview of the current state of knowledge regarding the results of different omics studies that have investigated the MHO phenotype. In some cases, because of the lack of genome-wide analyses, the results of targeted studies are presented. We believe that this multi-level approach will help to better understand the mechanisms behind healthy obesity. With the recent progress in technology, it is now possible to study processes on different molecular levels in detail and on a wide scale (Figure 2). Therefore, we performed extensive searches in the PubMed database for articles that were published between 2010 and 2020. The following search terms in a variety of combinations were used: ‘healthy obesity’, ‘MHO’, ‘metabolically healthy obese’, ‘genomics’, ‘transcriptomics’, ‘gene expression’, ‘proteomics’, ‘metabolomics’, ‘gut microbiota’, ‘epigenomics’, and ‘miRNA’.

GENOMICS

It is well established that genetic factors contribute to the development of obesity[24]; however, their role in metabolic health deterioration in obese individuals is still being clarified[21]. Even though genetic predisposition cannot be changed, being aware of the presence of certain variants allows the modification of environmental factors, which can delay or prevent the development of a disease or condition. Genome-wide association studies help to identify the link between a single nucleotide polymorphism (SNP) and a particular disorder[25].

In a study by Schlauch *et al*[26], 89 out of 905027 investigated SNPs showed an association with the MHO phenotype, which was defined by following characteristics: BMI ≥ 35 kg/m^2, normal blood pressure, normal fasting plasma glucose (less than 100

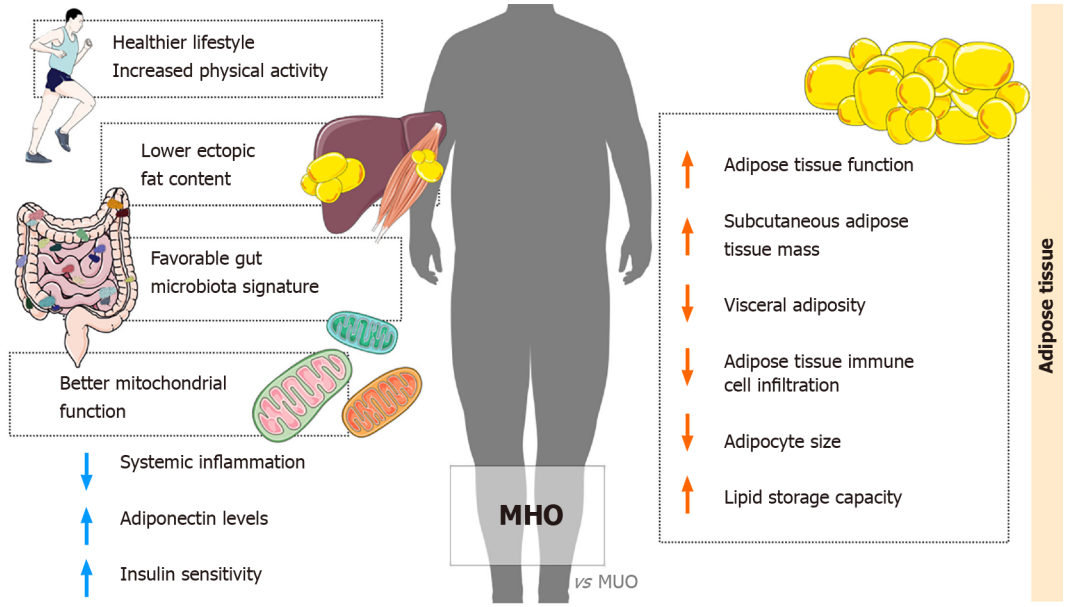

Figure 1 Differences in characteristics between metabolically healthy obese and metabolically unhealthy obese individuals. MHO: Metabolically healthy obese; MUO: Metabolically unhealthy obese.

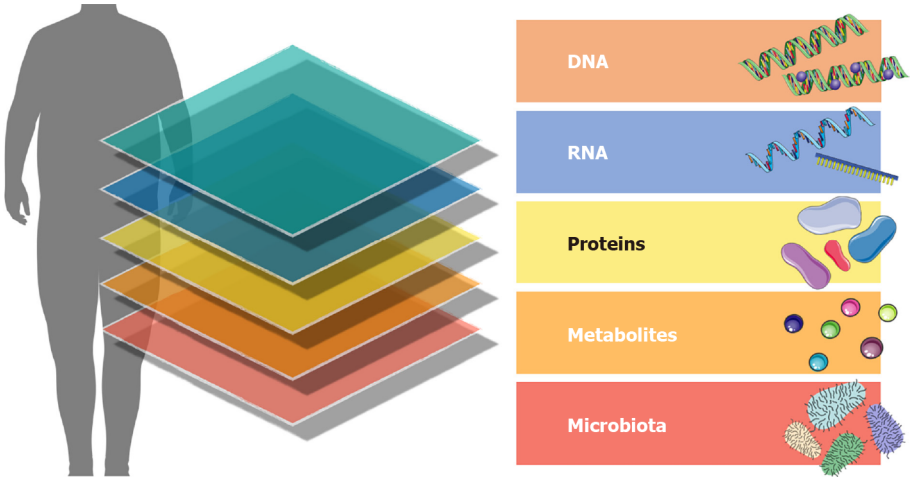

Figure 2 Different molecular levels targeted by a multi-omics approach.

mg/dL), and a desirable fasting lipid panel based on the National Cholesterol Education Panel Adult Treatment Panel III (NCEP ATP III) recommendations. Interestingly, all 89 SNPs identified in this study were located within intergenic regions, or non-coding regions, of their respective genes. Further analysis revealed that 12 genes associated with significant SNPs had been previously linked to obesity or cardiometabolic disease: *DPYD* to syndromic obesity, *KLHL6* to acute insulin response to glucose, *RTP4* to future incidence of hypertension, *PCDH7* to WHR, *FSTL4* to increased risk of stroke, *CSMD1* to MetS, *ITIH5* to BMI, *KCNQ1* to type 2 diabetes, *NBEA* to increased diastolic BP, *BBX* to type 1 diabetes, and *NTRK2* to obesity and depression. Furthermore, 31 polymorphisms were observed to be located close to at least one other SNP within the same gene or intergenic region, suggesting their special relevance according to the authors. A different study reported a significant association of *FTO* (rs1121980) and *TCF7L2* (rs7903146) genetic variants with maintaining metabolic health in obese women, who did not develop MetS according to joint interim statement criteria[27]. In addition, a genetic variant of *SLC39A8* (rs13107325) was found to have a statistically significant association with metabolic health status in a subgroup of postmenopausal women. The *TCF7L2* and *SLC39A8* genetic markers were shown to have a protective effect and help preserve metabolic health status, while the other marker was found to increase the risk of transitioning from the MHO

to MUO phenotype. The same *FTO* genetic marker (rs1121980) was demonstrated to have a significant association with the MUO but not MHO phenotype in a previous genotyping study, which also applied joint interim statement MetS definition[28].

Gao *et al*[29] investigated the association of SNPs of the *MC4R* gene with the MHO and MUO phenotypes in a Chinese population and found that rs2331841 increased the genetic risk of the MUO phenotype. Metabolically healthy subjects were defined as having less than two of the metabolic abnormalities in the NCEP ATP III criteria, while metabolically abnormal patients had at least two abnormalities. Based on the results of their study, the authors suggested that the rs2331841 SNP was associated with not only obesity but also metabolic abnormalities in obese subjects. Another group of researchers focused on the lactoferrin (*LTF*) gene-related polymorphisms and observed significant differences in the genotype frequencies of *LTF* rs2239692 between MHO and MUO individuals[30]. The CT variant of *LTF* rs2239692 was found to significantly decrease the risk of MetS development in obese individuals. Two other studies have reported an association of the adiponectin rs2241766 genetic variant with metabolic health phenotypes[31,32]. One reported more than a two-fold increase in the risk of developing metabolic abnormalities in the presence of the T45T genotype, while the second one found that the adiponectin T45G polymorphism was associated with the progression from the MHO to MUO phenotype. Both studies used International Diabetes Federation (IDF) MetS criteria to identify metabolically healthy individuals. The genetic variants associated with the MHO phenotype are presented in Table 1.

TRANSCRIPTOMICS

Gene expression

In recent years, transcriptome profiling has become one of the most common approaches for investigating diseases on a molecular level[33]. Gene expression profiling provides valuable information about any alterations in biological processes, and this information is crucial for not only understanding the underlying mechanisms but also making a molecular diagnosis and selecting a clinical therapy for the condition under study. The methods most widely used in gene expression studies are real-time reverse transcription polymerase chain reaction (RT-PCR), microarrays, and next-generation sequencing. Only a few research groups have applied a genome-wide approach to identify gene expression alterations in the MHO phenotype (Table 2). Gaye *et al*[34] performed whole blood transcriptome sequencing on samples from 8 MHO and 21 MUO individuals. The top 10 of the 149 enriched GO terms were related to mRNA translation processes. The canonical pathway analysis conducted in QIAGEN's Ingenuity Pathway Analysis (IPA) revealed significant enrichment in EIF2 signaling, regulation of eIF4 and p70S6K signaling, and mTOR signaling pathways. Of note, the top differentially expressed genes were ribosomal protein genes. The authors stated that the MUO subjects exhibited higher levels of ribosomal and endoplasmic reticulum (ER) stress and inflammation than the MHO individuals. They also noted that the TG/HDL-C ratio was possibly the major driver of the altered expression of ribosomal protein genes in these two groups. The authors proposed controlling ER and ribosomal stress and keeping the TG/HDL-C ratio in the proper range as potential strategies to prevent the development of an unfavorable metabolic profile in obese individuals.

We found that similar pathways were enriched in our recent transcriptomic study of different metabolic health phenotypes[35]. The IPA core analysis of differentially expressed genes between MHO and MHNW subjects revealed significant enrichment in EIF2 signaling, regulation of eIF4 and p70S6K signaling, oxidative phosphorylation, mitochondrial dysfunction, and mTOR signaling canonical pathways. Moreover, a vast majority of the genes within each of the identified pathways were upregulated. We also compared gene expression patterns between MUO and MHO individuals and found enrichment in canonical pathways associated with processes of inflammation, coagulation and thrombosis, hepatic fibrosis, and atherosclerosis signaling. Another study used whole genome microarrays to evaluate gene expression in the peripheral blood of obese individuals with and without metabolic disturbances[36]. The data analysis revealed enrichment in genes involved in lipid metabolism, carbohydrate metabolism, protein synthesis, and the activation, signaling, and function of cells. Another microarray study analyzed gene expression in abdominal subcutaneous adipose tissue samples from MHO and MUO subjects[37]. The authors performed unsupervised hierarchical clustering of 1595 obesity-associated transcripts and identified two obesity subtypes among the study participants. Differentially expressed

Table 1 Single nucleotide polymorphisms associated with metabolically healthy obese phenotype

Nearest gene	SNPs	Ref.
ADIPOQ	rs2241766	Berezina *et al*[31] and Chang *et al*[32]
MC4R	rs2331841	Gao *et al*[29]
LTF	rs2239692	Jamka *et al*[30]
FTO	rs1121980	Gharooi Ahangar *et al*[27]
TCF7L2	rs7903146	Gharooi Ahangar *et al*[27]
SLC39A8	rs13107325	Gharooi Ahangar *et al*[27]
RTP4	rs9028, rs9843429	Schlauch *et al*[26]
CDH18	rs1022207, rs2967027	Schlauch *et al*[26]
FST/LOC257396	rs37785, rs10461563	Schlauch *et al*[26]
FSTL4/WSPAR	rs7719102, rs4246020	Schlauch *et al*[26]
LOC107986637	rs11753543, rs9384860	Schlauch *et al*[26]
TRPS1	rs2245221, rs2737214, rs2737215	Schlauch *et al*[26]
DLG2	rs7131460, rs12275254	Schlauch *et al*[26]
TOX2	rs766622, rs6065690, rs6093921	Schlauch *et al*[26]
FAM19A2/SLC16A7	rs4143650, rs6581305	Schlauch *et al*[26]
LOC101927367/LOC105371833	rs11079177, rs12601773	Schlauch *et al*[26]
LOC105371989	rs206549, rs206547	Schlauch *et al*[26]
LOC107986666	rs9295227, rs9458896, rs6928576, rs6902153, rs10945918, rs7748991, rs9356148	Schlauch *et al*[26]

SNPs: Single nucleotide polymorphisms.

genes between the two groups were mainly enriched in CVD pathways (occlusion of artery, vascular disease, peripheral arterial occlusive disease) and immune and inflammatory response pathways (complement system, TREM1 signaling, interleukin-8 signaling).

Targeted gene expression analyses of the MHO phenotype have been much more common. In one study, five out of six investigated mitophagy genes showed no significant differences in expression between MHO and metabolically healthy non-obese subjects[38]. The authors concluded that unaltered mitophagy allowed the control of oxidative stress and inflammation, leading to improved mitochondrial function and preserved insulin sensitivity in MHO subjects. An earlier study reported lower levels of oxidative and ER stress in MHO individuals based on the results of a gene expression experiment focusing on genes involved in the regulation of the unfolded protein response[39]. Another research group investigated a set of proinflammatory genes and found no significant differences between abdominally obese MHO and MUO subjects[40,41]. The two groups were matched for abdominal fat, and because of this matching, the authors suggested that the expression levels of Toll-like-receptor (TLR) 2, TLR4, TRIF, MyD88, and nuclear factor-κappa beta (NF-κB) were related more to abdominal obesity than to metabolic health status. Telle-Hansen *et al*[42] analyzed the expression of selected genes involved in lipid uptake, transport, lipolysis, lipogenesis, and fatty acid oxidation and found reduced gene expression of *UCP2*, *LIPE*, and *PPARG* in the MUO group compared to that in the MHO group, while no differences were observed between MHO and MHNW individuals. A different research group analyzed the expression of inflammatory and matrix remodeling genes in visceral adipose tissue (VAT) and liver tissue samples from MHO and MUO subjects and found similar gene expression profiles between the groups in both tissues[43].

miRNAs

miRNAs are short non-coding RNA molecules consisting of around 22 nucleotides[44]. They have been proven to be important gene expression regulators, and most human protein-coding genes are regulated by at least one miRNA[45]. They exert their function

Table 2 Studies investigating genome-wide gene expression levels in metabolically healthy obese individuals

Biological material	Method	Study design	Sample size	Metabolic health definition	Main findings	Ref.
Whole blood	RNA-seq	MHO *vs* MUO	8/21	All conditions must be met: BP ≤ 130/85 mmHg, no medication; FPG ≤ 100 mg/dL, no medication; HOMA-IR ≤ 5.1; TG/HDL ≤ 1.65 for males, TG/HDL ≤ 1.32 for females; hsCRP ≤ 0.3 mg/dL	Enrichment in pathways: EIF2 signaling, eIF4 and p70S6K signaling, mTOR signaling. Enrichment in GO terms related to mRNA translation processes. Ribosomal protein genes among top differentially genes	Gaye *et al*[34]
Whole blood	RNA-seq	MHO *vs* MUO	8/8	No MetS, according to harmonized MetS definition	Enrichment in pathways: granulocyte/agranulocyte adhesion and diapedesis, coagulation system, intrinsic prothrombin activation pathway, atherosclerosis signaling, integrin signaling, binding and aggregation of blood cells	Paczkowska-Abdulsalam *et al*[35]
Whole blood	RNA-seq	MHO *vs* MHNW	8/8	No MetS, according to harmonized MetS definition	Enrichment in pathways: EIF2 signaling, eIF4 and p70S6K signaling, mTOR signaling, oxidative phosphorylation, mitochondrial dysfunction, vascular/arterial disease	Paczkowska-Abdulsalam *et al*[35]
PBMCs	Microarray	MHO *vs* MHNW	17/15	No MetS, according to NCEP ATP III MetS definition	Enrichment in pathways: carbohydrate metabolism, lipid metabolism, protein synthesis, amino acid metabolism, cell morphology, death and survival, cell-to-cell signaling and interaction, cellular development, movement, growth and proliferation	de Luis *et al*[36]
aSAT	Microarray	MHO *vs* MUO	16/14	MHO group identified through unsupervised hierarchical clustering of 1595 obesity-associated transcripts	Enrichment in pathways: complement system, TREM1 signaling, IL-8 signaling, actin cytoskeleton signaling, vascular disease, occlusion of artery	Das *et al*[37]

aSAT: Abdominal subcutaneous adipose tissue; PBMCs: Peripheral blood mononuclear cells; NCEP ATPIII: National Cholesterol Education Program Adult Treatment Panel III; BP: Blood pressure; FPG: Fasting plasma glucose; HOMA-IR: Homeostatic model assessment for insulin resistance; TG: Triglycerides; HDL: High-density lipoprotein; CRP: C-reactive protein; MHO: Metabolically healthy obese; IL: Interleukin.

through binding to target mRNAs, which results in mRNA translational repression or transcript degradation, depending on the degree of base-pairing complementarity[46]. Specific miRNA signatures have been described in many diseases, including obesity, type 2 diabetes, and CVD[22]. To date, however, only a few studies have investigated miRNAs in the MHO phenotype. The molecules that have been identified in MHO subjects by different research groups are presented in Table 3.

In a recent study, Yang *et al*[47] used microarrays to analyze miRNA levels in plasma samples from six young MHO individuals and six healthy controls. Individuals with diabetes mellitus, hypertension, hyperlipidemia, coronary artery disease, valvular or congenital heart disease, cardiomyopathy, atrial fibrillation, and heart failure were excluded from the study. A distinct miRNA profile was identified for the MHO phenotype. Gene ontology analysis revealed the highest enrichment in the following terms: cell-cell adhesion, translational initiation, and apoptotic process among biological processes; nucleoplasm and nucleus among cellular components; and protein binding, DNA binding, and protein serine/threonine kinase activity among molecular functions. The expression levels of six of the top ten up- or downregulated miRNAs were further confirmed in a group of 600 MHO subjects in a RT-PCR experiment. MiRNA-500, miRNA-454, and miRNA-320a were found to be downregulated in MHO individuals, while miRNA-21, miRNA-148a, and miRNA126 were found to be upregulated. The authors reported that high circulating miRNA-21 Levels were associated with impaired diastolic function as well as increased cardiac fibrosis markers *via* the TGF-β1/Smad signaling pathway. A different set of miRNAs was identified in another study, which screened 179 serum miRNAs in a group of MHO and MUO individuals[48]. The authors applied a stringent inclusion criteria of three distinct definitions of MHO: the basic definition; the modified Wildman definition; and the most stringent definition which adds an inflammation marker to all parameters included in the modified Wildman definition. One of the eight differentially expressed miRNAs, identified in this study, remained significant after validation in an independent sample of 98 obese subjects with/without metabolic abnormalities. According to the authors, the differences in miRNA-374-5p expression between the MHO and MUO phenotypes were related to the TG/HDL-C ratio. Based

Table 3 Different expression of micro-RNAs identified in metabolically healthy obese individuals

miRNA	Upregulation/ Downregulation	Sample size (MHO/MHNW)	Biological material	Ref.
MHO *vs* MHNW				
hsa-miR-500[1]	↓	6/6	Plasma	Yang *et al*[47]
hsa-miR-454[1]	↓			
hsa-miR-142	↓			
hsa-miR-320a[1]	↓			
hsa-miR-107	↓			
hsa-miR-34a	↑			
hsa-miR-21[1]	↑			
hsa-miR-99b	↑			
hsa-miR-148a[1]	↑			
hsa-miR-126[1]	↑			
MHO *vs* MUO				
hsa-miR-223-3p	↓	10/10	Serum	Doumatey *et al*[48]
hsa-miR-374a-5p[1]	↑			
hsa-miR-10b-5p	↑			
hsa-miR-26b-5p	↑			
hsa-let-7d-3p	↑			
hsa-miR-29a-3p	↑			
hsa-miR-342-3p	↑			
hsa-miR-16-2-3p	↑			
hsa-miR-503	↑	34/21	Serum	Yue *et al*[49]

[1]Validated with real-time reverse transcription polymerase chain reaction in an independent cohort. miRNA: Micro-RNAs; MHO: Metabolically healthy obese; MHNW: Metabolically healthy normal weight; MUO: Metabolically unhealthy obese.

on an analysis in an independent cohort, they also stated that miRNA-374-5p may modulate CCL2 expression, which is a pro-inflammatory marker, upstream of the pathway leading to dyslipidemia in obesity.

A more recent study applied a targeted RT-PCR approach and found significantly higher serum miRNA-503 Levels in MHO and MHNW subjects than in MUO individuals[49]. It was found that miRNA-503 negatively correlated with metabolic abnormalities in obese subjects, and the authors concluded that miRNA-503 can serve as a biomarker to distinguish between the MHO and MUO phenotypes, which were defined according to the 2007 Joint Committee for Developing Chinese Guidelines. Another study defined good metabolic health as being IS despite concurrent obesity[50]. Fifteen miRNAs were observed to be differentially expressed in subcutaneous white adipose tissue between IS and insulin resistant (IR) obese women. In further functional *in vitro* studies, the authors concluded that miRNA-143-3p and miRNA-652-3p might enhance insulin-stimulated glucose incorporation into lipids in human fat cells. Jones *et al*[51] analyzed 175 miRNAs in the plasma of IS and IR obese women and healthy controls. MiRNA-335 and miRNA-423-5p were found to be differentially expressed between the IR and IS groups. The authors also compared the miRNA profiles of the IS obese and healthy control groups and reported the top ten up- and downregulated molecules with different expression levels in each of the groups.

DNA METHYLATION

DNA methylation is an epigenetic mechanism involving the covalent addition of methyl groups to nucleotide bases, usually cytosines in CpG dinucleotide sequences. It

affects gene expression levels by regulating interactions with transcriptional activators and repressors, as well as chromatin remodeling enzymes[52]. This heritable genetic marker is considered to play a major role in the epigenetic suppression of the transcription process. Differentially methylated regions have been described in a variety of conditions, including type 2 diabetes, cancers, and autoimmune and neurological disorders[53]. Only a few studies, however, have investigated this type of DNA modification and its association with the MHO phenotype.

A group led by Turcot analyzed the methylation levels of long interspersed nuclear element 1 (*LINE-1*) repetitive elements in VAT samples from obese individuals with and without MetS[54]. As *LINE-1* is considered to be a marker of genome-wide methylation, the authors concluded that lower global DNA methylation levels were associated with a greater risk of MetS in the presence of obesity. A year later, the same group reported that they found no association between dipeptidyl peptidase-4 (*DPP4*) methylation levels and metabolic health status[55]. In their study, Guénard *et al*[56] applied a genome-wide approach to analyze altered biological pathways in VAT samples from obese men with and without metabolic abnormalities, which were defined by NCEP ATP III MetS definition. Using differential methylation analysis, they identified 8578 CpG sites with significant differences between the groups. Some of them were located within genes previously described as being related to processes of cellular growth and proliferation (*PRKCA, PPP2R2B*), lipid metabolism (*FASN, LRP1B, PLA1A*), or inflammation (*IL17RA*). Of note, the *RPTOR* gene, which regulates cell growth in response to nutrient and insulin levels, was found to contain 28 differentially methylated sites[56]. The subsequent enrichment analysis revealed pathways related to hepatic cholestasis, renin-angiotensin signaling, cell cycle regulation (cdc42 signaling, inositol phosphate metabolism), inflammation and immunity (antigen presentation pathway, autoimmune diseases signaling), and structural components of the cell membrane (glycerophospholipid/phospholipid metabolism).

PROTEOMICS

Proteomics enables the genome-wide identification and quantification of all proteins present at a certain moment in a cell or tissue of interest. It can provide valuable insight into the molecular basis of a studied condition, disease, or phenotype at the protein level. Proteomics-based approaches are widely applied in different research areas, and they often lead to the discovery of novel biomarkers or drug targets[57]. The proteome of the MHO phenotype has also been studied by a few research groups (Table 4). In one study, a serum analysis of 20 African-American women was performed, and 20 differentially expressed proteins were identified between the MHO and MUO groups[58]. Metabolically healthy subjects fulfilled criteria of all three distinct definitions of the MHO phenotype: the basic definition; the modified Wildman definition; and the most stringent definition which adds an inflammation marker to all parameters included in the modified Wildman definition. Pathway analysis revealed enrichment in inflammatory and lipid pathways, including LXR/RXR (liver X receptor/ retinoid X receptor) and FXR/RXR (farnesoid X receptor/retinoid X receptor) activation, atherosclerosis signaling, acute phase response signaling, and the complement system. Overall, lower levels of pro-inflammatory and higher levels of anti-inflammatory markers were observed in the MHO status than in the MUO status. A more recent study investigated changes in the urinary proteome of 18 obese individuals with different metabolic statuses defined by the IDF MetS criteria[59]. The authors detected 54 proteins with altered expression, and most were related to the NF-κB and p38 mitogen-activated protein (MAP) kinase pathways. The MUO study participants were found to have a higher abundance of proteins involved in inflammation (FIBA, TRFE, KNG1) and insulin resistance (ARL15, RET4) than the MHO individuals. In another study, 28 differentially expressed proteins were identified in VAT samples obtained from 18 patients undergoing bariatric surgery who had previously been divided into MHO and MUO groups according to the IDF MetS definition[60]. The differentially expressed proteins belonged to three functional categories: protein and lipid metabolism, cytoskeleton, and regulation of other metabolic processes. The top overrepresented IPA canonical pathways included death receptor signaling, coagulation system, acute phase response signaling, Rho GDI signaling, and NRF2 (nuclear factor erythroid 2-related factor 2)-mediated oxidative stress response. The proteins that were found to be upregulated in MHO individuals were mainly cytoskeletal and antioxidant proteins, as well as proteins involved in

Table 4 Proteins with altered expression in metabolically healthy obese compared to metabolically unhealthy obese individuals

Biological material	Over-expressed	Under-expressed	Top enriched pathways	Ref.
Serum	APOB, AHSG, SERPINC1, APOA4, SERPING1, RBP4, ITIH2, GSN, HRG, ITIH1, GC, C7	HBA1, HPR, HBB, CFB, ITIH4, CRP, PON1, C4A	LXR/RXR activation, FXR/RXR activation, acute phase response signaling, complement system, atherosclerosis signaling, IL-12 signaling and production in macrophages, production of nitric oxide and reactive oxygen species in macrophages, clathrin-mediated endocytosis signaling, extrinsic prothrombin activation pathway, intrinsic prothrombin activation pathway, coagulation system	Doumatey *et al*[58]
Urine	RASN, IGHG2, K1C10, VTDB	ACOT2, ARL15, APC4, APC7, APOA1, DYH3, FIBA, C1GLT, HIX, ITIH4, KNG1, P3H2, AMBP, COO33, RET4, TRFE, ZFP2, ZN568, ZN655	LXR/RXR activation, FXR/RXR activation, acute phase response signaling, clathrin-mediated endocytosis signaling, atherosclerosis signaling, IL-12 signaling and production in macrophages, coagulation system, intrinsic prothrombin activation pathway, production of nitric oxide and reactive oxygen species in macrophages, systemic lupus erythematosus signaling	Benabdelkamel *et al*[59]
VAT	ANXA5, ACTG, ACTB, LEG1, GPDA, APOA1, CO6A1, SBP1, CATA, TO20L, BRE1A, RNA58, SOX21	POTEE, SPTN4, GDIR1, TTHY, HSP1, PPIA, UPAR, PAI1, BLVRB, ERI2, YQ019	death receptor signaling, coagulation system, acute phase response signaling, RhoGDI signaling, NRF2-mediated oxidative stress response	Alfadda *et al*[60]

IL: Interleukin; VAT: Visceral adipose tissue; LXR: Liver X receptor; RXR: Retinoid X receptor, FXR: Farnesoid X receptor; NRF2: Nuclear factor erythroid 2-related factor 2; RhoGDI: Rho GDP-dissociation inhibitor.

mitochondrial import and transcriptional activity. The MUO individuals, on the other hand, were found to exhibit the upregulation of proteins that increase metabolic dysfunction in the ECM, mitochondria, and lipid droplets. The authors noted that the differentially expressed proteins were involved in complex pathways linked to insulin dysregulation. Doulamis *et al*[61] used a targeted proteomic approach to measure 30 potential biomarkers in serum and VAT samples from 28 obese patients undergoing bariatric surgery. The metabolic health status was defined as the presence (MUO) or absence (MHO) of comorbidities such as hypertension, dyslipidemia and diabetes mellitus. Compared to the levels in MUO individuals, six downregulated proteins in VAT (TWEAK, TRAIL, GDF-15, RETN, MMP-9, ICTP) and four downregulated proteins in serum (interleukin-20, PROK-1, TWEAK, CCL-3) were identified in MHO individuals. Those with the MUO phenotype were found to exhibit increased inflammation and higher levels of pro-inflammatory markers not only locally in adipose tissue but also systemically in the peripheral blood.

METABOLOMICS

Metabolomics is an emerging technology used to analyze all small molecule compounds that are products or substrates of chemical reactions within a biological system (cell, tissue, or organism)[62]. It provides comprehensive information on the activity and status of cellular and organismal metabolism and is therefore widely applied in different settings to identify metabolic disturbances underlying a disease, novel biomarkers, or new therapeutic targets[63]. The analysis of the molecular composition of a sample is carried out through the use of nuclear magnetic resonance (NMR) or mass spectrometry (MS). Both liquid and gas chromatography are applied to separate metabolites[62].

Several research groups that have investigated the MHO phenotype have performed metabolomic profiling experiments using NMR technology. A study on overweight and obese women with and without MetS in Finland found significant differences in branched-chain amino acids (BCAAs), aromatic amino acids (AAAs), orosomucoid, several species of fatty acids, and phospholipids between metabolically healthy and MetS study participants[64]. Of note, orosomucoid, BCAAs, and AAAs were associated with all MetS risk factors. A more recent study identified higher levels of alanine, glutamine, proline, asparagine, L-glutathione reduced, betaine, taurine, choline, 2-aminobutyrate, tagatose, and 2-oxoglutarate in MHO individuals than in MUO individuals[65]. Additionally, lower levels of L-alpha-phosphatidylinositol and D-sphingosine were observed in MHO subjects. A pathway enrichment analysis that

compared MUO and MHO subjects revealed alterations in the urea cycle, ammonia recycling, aspartate metabolism, and glycine and serine metabolism, among other pathways (Table 5).

A research group led by Telle-Hansen *et al*[42] reported that the concentrations of very low-density lipoprotein, intermediate-density lipoprotein, and low-density lipoprotein subclasses were, overall, significantly higher in MUO individuals than in MHO individuals, with the MHNW group having the lowest values. In addition, the levels of HDL subclasses were lower in MUO and higher in MHNW individuals than in the MHO group; together, these results indicate that MHO subjects have an intermediate CVD risk profile compared to those of MUO and MHNW individuals. Similarly, an earlier study that used gas chromatography-MS analysis reported that the MHO group had an intermediate serum amino acid profile compared to those of MHNW and MUO subjects[66]. Even though most individual amino acids did not differ significantly between the MHO and MUO groups, several of them, including glycine, reflected the intermediate cardiometabolic profile of the MHO phenotype. Overall, the results suggested improved insulin sensitivity in the MHO group compared to that in the MUO group, as well as differences between the two groups in the availability of metabolites that enter the TCA cycle. Hydroxyproline concentration together with the ratios of other amino acids were proposed as biomarkers for distinguishing between the MHO and MUO phenotypes.

In their study, Chen *et al*[67] identified a group of serum metabolites, including L-kynurenine, glycerophosphocholine, glycerol 1-phosphate, glycolic acid, tagatose, methyl palmitate, and uric acid, that differed significantly between the MHO and MUO groups. In addition, they found that several metabolic pathways, such as fatty acid biosynthesis, phenylalanine metabolism, propanoate metabolism, and valine, leucine, and isoleucine degradation, were altered between the two phenotypes. The authors reported an association of liver and mitochondria functions with metabolic disturbances in obese subjects. Another metabolomics study reported that the plasma metabolic profiles of MetS obese individuals correlated with the fulfillment of a number of criteria used to diagnose MetS[68]. Fifteen metabolites, including nucleosides, amino acids and derivatives, amino sugars, purine derivatives, and polyols, that could differentiate between metabolically healthy and unhealthy individuals were identified. These metabolites with altered concentrations belonged to numerous physiologically significant pathways, such as purine metabolism; valine, leucine, and isoleucine degradation; aminoacyl-tRNA biosynthesis; and tryptophan metabolism. A case control study that included over 100 MHO individuals reported that BCAAs, glutamic acid, tyrosine, and a specific pattern of lysophosphatidylcholines were associated with both the MHO and MUO phenotypes[69]. Interestingly, when the MUO and MHO phenotypes were directly compared, no significant differences in metabolites were found. Another study highlighted the importance of body fat distribution and performed a metabolomic analysis of serum samples from metabolically healthy peripherally obese and metabolically unhealthy centrally obese individuals[70]. The authors found that significantly higher levels of BCAAs (leucine, isoleucine, valine), propionylcarnitine (C3 acylcarnitine), and alpha-aminoadipic acid could distinguish metabolically unhealthy central obesity from metabolically healthy peripheral obesity.

To better understand the role of visceral fat in the development of metabolic abnormalities, Candi *et al*[71] analyzed the metabolomic profiles of VAT samples from MHO and MUO subjects. They found that the state of pathological obesity was associated with the increased metabolism of γ-glutamyl amino acids, which are involved in glutathione metabolism and the response to oxidative stress, and plasmalogens, which contribute to insulin resistance and hypertension[72].

MICROBIOME

Increasing evidence suggests that the human gut microbiota has a significant impact on maintaining immune and metabolic homeostasis and protecting against pathogens[73]. Changes in the composition of intestinal bacteria have been reported in a variety of disorders, including neurological, cardiovascular, and respiratory illnesses[74]. The most popular method for studying the human endogenous microbial community is 16S rRNA sequencing, which can effectively distinguish between different taxa[75]. To date, only a few research groups have investigated the association between gut microbiota and the MHO phenotype. Kim *et al*[76] profiled fecal microbiota from over 700 overweight and obese Korean individuals who had no metabolic

Table 5 Differentially regulated pathways between metabolically healthy obese and metabolically unhealthy obese groups identified by metabolomics studies

Study	Chen *et al*[67], 2015	Zhong *et al*[68], 2017	Candi *et al*[71], 2018	Chashmniam *et al*[65], 2020
Biological material	Plasma	Plasma	VAT	Serum
Method	LC-MS, GC-MS	LC-MS/MS	LC-MS/MS	NMR
MHO definition	No MetS, according to NCEP ATP III MetS definition	No MetS, according to harmonized MetS definition	No MetS, according to NCEP ATP III MetS definition	No MetS, according to NCEP ATP III MetS definition
MHO (*n*)	34	43	18	21
MUO (*n*)	34	26	18	21
Affected pathways	Fatty acid biosynthesis; Phenylalanine metabolism; Propanoate metabolism; Valine, leucine and isoleucine degradation; Pyrimidine metabolism; Citrate cycle (TCA cycle); Galactose metabolism; Glyoxylate and dicarboxylate; and Tryptophan metabolism	Purine metabolism (*i.e.*, urate); Valine, leucine and isoleucine degradation; Aminoacyl-tRNA biosynthesis; Tryptophan metabolism; Cysteine and methionine metabolism; Lysine degradation; Pyrimidine metabolism; Arginine and proline metabolism; Glycine, serine and threonine metabolism; Taurine and hypotaurine metabolism; Alanine, aspartate and glutamate metabolism; Pantothenate and CoA biosynthesis	Ceramide metabolism; Phosphatidylserine; Fatty acid, dicarboxylate; Glutathione metabolism; Lysoplasmalogen; Lysolipid; Aminosugar metabolism; Gamma-glutamyl amino acid; Pyrimidine metabolism, uracyl containing; Plasmalogen; Glycerolipid metabolism; Sphingolipid metabolism; Phopsholipid metabolism; Fructose, mannose, and galactose metabolism	Urea cycle; Ammonia recycling; Aspartate metabolism; Glycine and serine metabolism; Glucose-alanine cycle; and Arginine and proline metabolism

LC: Liquid chromatography; GC: Gas chromatography; MS: Mass spectrometry; NCEP ATP III: National Cholesterol Education Program Adult Treatment Panel III; TCA: Citric acid cycle; MetS: Metabolic syndrome; NMR: Nuclear magnetic resonance; MHO: Metabolically healthy obese; MUO: Metabolically unhealthy obese.

abnormality (metabolically healthy) or had at least one metabolic abnormality (metabolically unhealthy). To define metabolic abnormalities the NCEP ATP III criteria were used. Genera such as *Oscillospira* and *Clostridium* were found to be significantly more abundant in metabolically healthy individuals, and the results were similar for the family Coriobacteriaceae within *Actinobacteria* and the family Leuconostocaceae within Firmicutes. On the other hand, metabolically unhealthy individuals were found to have an increased abundance of Fusobacteria. Interestingly, no differences in the Firmicutes/Bacteroidetes ratio between the two groups were observed. Of note, the microbial profile of metabolically healthy overweight/obese subjects was closer to that of lean individuals than to that of metabolically unhealthy overweight/obese individuals. A study on obese Mexican women with and without MetS reported that three genera and families were significantly enriched in the obese without MetS group: *Roseburia*, *Succinivibrio*, and S24-7 (all were three-fold more abundant than in the MetS obese and control groups)[77]. Another study, in which the NCEP ATP III MetS definition was applied to define the metabolic status of older Irish individuals, reported no differences in the diversity, richness, or taxonomy between the MHO and MUO groups[78]. A different approach was applied by Kashtanova *et al*[79], who analyzed the association of the gut microbiota composition with individual cardiovascular risk factors. An increase in the abundance of certain genera was observed depending on the type of metabolic abnormality: *Blautia* in cases of impaired carbohydrate metabolism and *Prevotella* in individuals with elevated BP and obesity. The abundance of *Serratia* increased together with a number of cardiovascular risk factors, whereas an inverse relationship was observed between the abundance of *Oscillospira* and abdominal obesity.

CONCLUSION

As evidenced by the findings discussed in this review, over the past several years, enormous progress has been made regarding the molecular characterization of different metabolic health phenotypes. The studies conducted to date are a source of valuable information on the protective mechanisms underlying the MHO phenotype or the mechanisms responsible for metabolic health deterioration and the transition from the MHO to MUO phenotype. Genomic studies have identified genetic variants

that are related to increased adiposity with a favorable metabolic profile. Many of the identified loci are located near genes previously reported to be involved in insulin signaling, insulin resistance, fat distribution, and adipogenesis. The results of transcriptomic analyses suggest that mitochondrial dysfunction, ER and oxidative stress, pathological changes in the liver, and activated atherosclerotic processes contribute to the development of metabolic abnormalities. Moreover, miRNA profiling studies have identified several molecules that are characteristic of the MHO phenotype, some of which are involved in processes such as cell-cell adhesion, translational initiation, and the apoptotic process. Epigenomic analyses have revealed differences in the methylation of sites within genes involved in lipid metabolism, inflammation, and cellular growth. Similar pathways have been found to be altered within the proteomes of different metabolic health phenotypes. Metabolomics has been used to confirm the important role of liver and mitochondria functions in metabolic disturbances, while significant differences in the gut microbiota composition have been found between MHO and MUO individuals.

Nevertheless, multiple issues still need to be addressed in the near future to take full advantage of all the research done in this field. The inconsistent or even conflicting results among some studies underscore the need for a unique metabolic health definition that is agreed upon by everyone in the research community. There must be consensus regarding not only the criteria and their cut-off values but also the number of risk factors that determine the MHO phenotype. Until then, the interpretation and comparison of the results from different study groups will remain difficult, if not impossible. Another challenge is to go beyond pilot molecular studies with small numbers of participants and develop national and international collaborative networks. Larger sample sizes should address the issues of heterogeneity among study participants, as well as difficulties in the recruitment of the most MHO individuals, who do not exhibit any of the risk factors despite their obesity. Another issue that needs to be considered is that only a few studies on metabolic health have applied the global analysis approach in the form of omics technologies, likely because of the high cost combined with the limited funds of pilot studies. Ideally, a large-scale study on universally defined metabolic health phenotypes should be carried out, implementing all omics technologies and therefore exploring metabolic function comprehensively at all possible levels: genomic, transcriptomic, epigenomic, proteomic, and metabolomic, as well as that of the gut microbiota. The markers identified with different omics methods can then be fitted into a multi-omics framework, revealing the interplay between molecules or pathways and the roles of different factors in mechanisms underlying metabolic health deterioration.

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MINIREVIEWS

Malfunction of outer retinal barrier and choroid in the occurrence and progression of diabetic macular edema

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Abstract

Diabetic macular edema (DME) is the most common cause of vision loss in diabetic retinopathy, affecting 1 in 15 patients with diabetes mellitus (DM). The disruption of the inner blood-retina barrier (BRB) has been largely investigated and attributed the primary role in the pathogenesis and progression in DME, but there is increasing evidence regarding the role of outer BRB, separating the RPE from the underlying choriocapillaris, in the occurrence and evolution of DME. The development of novel imaging technologies has led to major improvement in the field of *in vivo* structural analysis of the macula allowing us to delve deeper into the pathogenesis of DME and expanding our vision regarding this condition. In this review we gathered the results of studies that investigated specific outer BRB optical coherence tomography parameters in patients with DM with the aim to outline the current status of its role in the pathogenesis and progression of DME and identify new research pathways contributing to the advancement of knowledge in the understanding of this condition.

Key Words: Diabetic macular edema; External limiting membrane; Hyperreflective foci; Inner segment/outer segment line; Optical coherence tomography; Outer retinal barrier

Core Tip: Progress in optical coherence tomography technology allowed the identification of new pathogenic pathways in diabetic macular edema (DME) involving the outer retina and underlying choroid. The presence of fluid in the subretinal space is suggestive for the alteration of the outer blood retinal barrier and responds better to

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intravitreal triamcinolone as compared to anti- vascular endothelial growth factor. The disruption of external limiting membrane (ELM) is associated with visual impairment being a predictor of poor outcomes following the treatment with triamcinolone. The integrity of ELM and of the inner segment/outer segment line was found to correlate positively with best corrected visual acuity in DME.

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INTRODUCTION

Diabetic macular edema (DME) is the most common cause of vision loss in diabetic retinopathy (DR)[1], affecting 1 in 15 patients with diabetes mellitus (DM)[2]. DME is the first cause of visual impairment within the group of working-age population in the developed countries[3]. The retina is one of the most metabolically active tissues in the organism, having high energetic demands. The complexity of the retinal activity requires a homeostatic microenvironment which is achieved by the functioning of two distinct blood-retina barriers, inner and outer. The disruption of the inner blood-retina barrier (BRB) has been largely investigated and attributed the primary role in the pathogenesis and progression in DME, but there is increasing evidence regarding the role of outer BRB, separating the retinal pigmented epithelium (RPE) from the underlying choriocapillaris, in the occurrence and evolution of DME[4]. The normal functioning of the RPE is crucial for the retina, as it removes the waste resulting from the phagocytosis of the photoreceptors' outer segments, it provides nutrients for the photoreceptors and it substitutes the lymphatics by pumping the fluid from the inner retina to the choriocapillaris[5]. In the diabetic retina, the highly hypoxic microenvironment leads to the over-expression of vascular endothelial growth factor (VEGF) with subsequent depletion of occludin and damage of the tight junctions between the RPE cells[6]. The RPE alteration in DME was demonstrated both morphologically and functionally. Thus, electron microscopy studies performed on the retinas with DME induced in animal models found shrank nuclei, reduced endoplasmic reticulum, infolding cell membrane, altered melanosome and loss of RPE cells[5]. Electroretinography on a mouse model showed the early decrease of c wave before the occurrence of any photoreceptor dysfunction[7]. The impact of VEGF on the RPE function was demonstrated on cell cultures: increase of VEGF led to the increase of transepithelial resistance (TER) which is a marker of RPE barrier's function. Following VEGF neutralization with an antibody, the RPE barrier's function recovered partially[8]. Following the analysis of the RPE cells' proteome, 62% of the proteins involved in the retinoic metabolism were found to be altered in diabetic eyes without retinopathy. Interestingly, these proteins were modified also in nonretinal tissue, proving that the alteration of RPE is part of the systemic effect of diabetes[9].

The development of novel imaging technologies has led to major improvement in the field of *in vivo* structural analysis of the macula allowing us to delve deeper into the pathogenesis of DME and expanding our vision regarding this condition[10]. Within the last decades through the implementation of specialized computer software systems and modern mathematical tools (fractal/multifractal and lacunarity analysis)[11,12] non-invasive predictive complementary tools were developed for an early diagnosis of patients with DME[13].

In this review we gathered the results of studies that investigated specific outer BRB optical coherence tomography (OCT) parameters in patients with DM with the aim to outline the current status of its role in the pathogenesis and progression of DME and identify new research pathways contributing to the advancement of knowledge in the understanding of this condition.

OUTER RETINAL BARRIER AND THE CHOROID IN THE PATHOGENESIS OF DME

The normal functioning of the retina is ensured by the blood-retinal barrier (BRB) which regulates the entry and exit of fluid and molecules, maintaining the retina transparent and dehydrated[14]. BRB is affected early in diabetic retinopathy (DR) which translates into increased vascular permeability and retinal edema[15]. BRB has two components, inner and outer.

Inner BRB is constituted by the tight junctions (zonula occludens) between the endothelial cells within the retinal vessel walls which allow interactions with pericytes and smooth muscle cells[16]. Pericytes have a critical role in maintaining of BRB, by liberating a lipid mediator which modulates it[17]. Retinal Müller cells and astrocytes stabilize the tight junctions between the endothelial cells[18], whereas microglia produces soluble factors which are important for vesicular communication[15].

Outer BRB is formed by the intercellular junction complex of the RPE. More specifically, the basolateral membrane of the RPE faces the Bruch's membrane, separating the RPE from the fenestrated endothelium of the choriocapillaris[19] (Figure 1).

These tight, adherens and gap junctions control the transport of fluids and solutes between the choroidal capillaries and the photoreceptor layers, thus maintaining the integrity of the retina[18]. It was proved that the RPE cells express major histocompatibility complex molecules, adhesion molecules and cytokines, thus playing an important role in immune processes[20]. Healthy RPE also regulates the retinal oxidative stress, therefore its malfunctioning reduces the level of antioxidants[19].

Even if the TER is much lower than the resistance of the inter-endothelial junctions at the inner retinal barrier, it efficiently prevents proteins and water from the choroid to enter the subretinal space and it allows water to exit towards the choroid following an osmotic gradient[14]. RPE dysfunction leads to the disruption of fluid transportation from the subretinal space towards the choriocapillaris which is translated into DME.

Hyperglycemia leads to the alteration of the junctional complexes at the level of the outer blood-retinal barrier subsequently to the activation of metalloproteinases by oxidative and nitrosative stress[14]. Since RPE is a highly polarized epithelium, any cytoskeletal alteration damages not only the junctions, but also the adequate distribution of membrane transporters leading to subretinal fluid accumulation[14].

Serous detachment of the macula which is very suggestive for the breakdown of the RPE barrier is observed in approximately one third of the DME cases[20,21].

Decanini *et al*[9] analyzed the RPE proteome in preretinopathic diabetic human donor eyes and identified significant biochemical changes preceding the clinically evident diabetic retinopathy. Some of the RPE altered proteins overlap with findings from other tissues affected by DM, but others were identified as novel biomarkers, such as proteins involved in retinoid metabolism, membrane dynamics and protein transport, proving that DM affects multiple cellular processes. Given the importance of RPE for the retinal functioning, these alterations may play a major role in the early pathogenesis of DR[9].

The external limiting membrane (ELM) is formed by tight-like and adherens junctions located at the interface between the retinal Müller glial cells and photoreceptor inner segments (Figure 2). Even if its role in the coordination of fluid movement around the macula is not fully understood, earlier studies showed that ELM serves as an important barrier to free protein diffusion across the retina[14]. Hyperglycemia alters the ELM by disrupting the tight junctions by the activation of PKCζ[22]. In DR the disruption of the junction protein complexes from the OLM translates clinically by lower visual acuity[23] and poor response to anti-VEGF therapy in DME[24,25].

Despite the information presented above, experimental studies proved that the RPE junction barrier is highly resistant to acute hypoxia/ischemia[26]. Besides, it is not known in which extent the RPE barrier is affected by retinal ischemia and the *in vivo* mechanisms involved in RPE dysfunction are not fully understood. For these reasons, the alterations of the outer retinal barrier drew less attention to the pathogenesis of DME compared to the ones of the inner retinal barrier[14].

The choroid is a highly vascularized and pigmented structure whose main role is to provide oxygen and nutrients to the intensely metabolic active outer retinal layers, namely the central avascular fovea and the prelaminary portion of the optic nerve[27]. Although the pathogenesis of DR is mainly attributed to the dysregulation of the retinal vasculature, there is evidence pointing to diabetic choroidopathy[28]. Histological studies of the choroid revealed atrophy of the choriocapillary

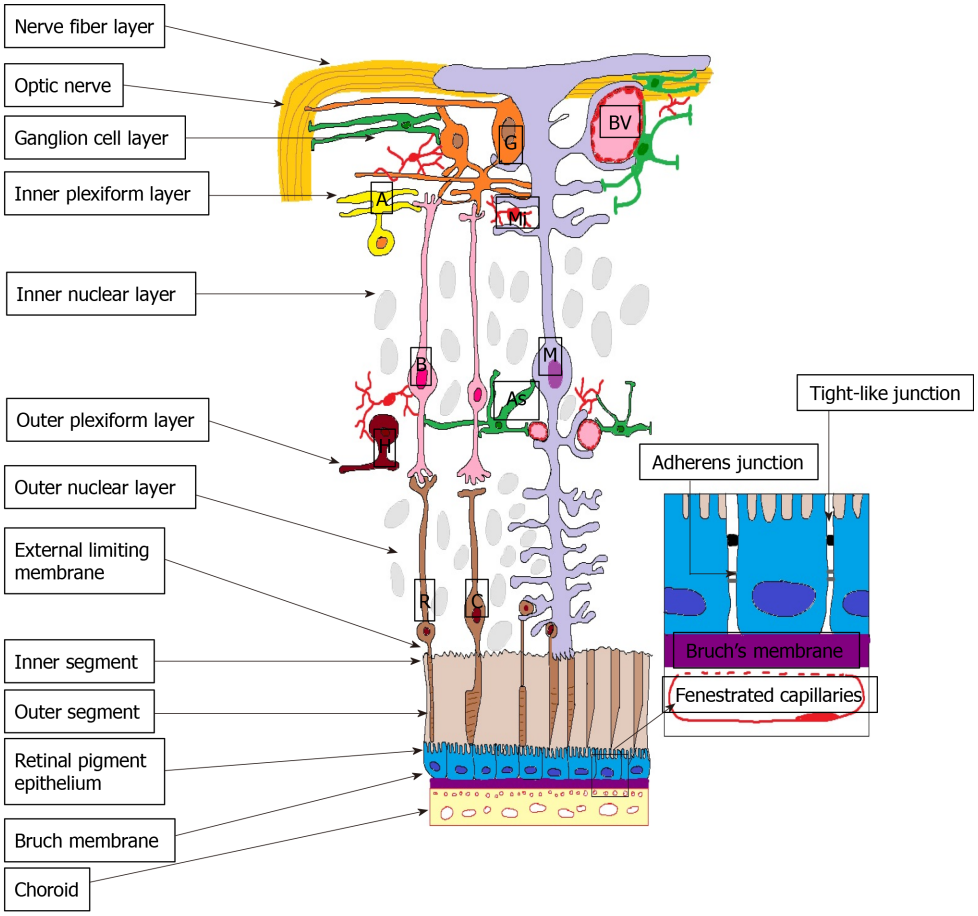

Figure 1 Outer blood-retina barrier. M: Müller cells; A: Amacrine cells; G: Ganglion cells; BV: Blood vessels; As: Astrocyte; B: Bipolar cells; R: Rods; C: Cones; H: Horizontal cells; Mi: Microglia; ON: Optic nerve; NFL: Nerve fibre layer; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; ELM: External limiting membrane; IS: Inner segment photoreceptors; OS: Outer segment photoreceptors; RPE: Retinal pigment epithelium; BM: Bruch's Membrane; Ch: Choroid.

endothelium, laminar deposits and narrowing of the luminal area in diabetic patients without DR[29,30], as well as basement membrane thickening, capillary dropout and choroidal neovascularization[28].

OPTICAL COHERENCE TOMOGRAPHY BIOMARKERS OF THE OUTER RETINA IN DME

Currently, precision retinology is in-conceivable without the use of OCT which allows early diagnosis, monitoring and individualization of treatment in patients with DM. The normal OCT aspect of the retinal layers is illustrated in Figure 3.

Serous retinal detachment

Based on the OCT appearance, three major types of DME were individualized: diffuse sponge-like thickening type, cystoid type (thickening of the fovea with intraretinal cysts) and serous retinal detachment (SRD) type (thickening of the fovea with subretinal fluid)[18,31,32].

Each of these lesions occurs in individual retinal layers, as follows: cystoid spaces are located mainly in the inner nuclear layer (INL) and outer plexiform layer (OPL); in SRD the extracellular fluid pools between the photoreceptors outer segments (PROS) and RPE; sponge-like retinal swelling at the fovea is identified in the OPL[33]. Whereas the sponge-like retinal swelling is frequently accompanied by abnormalities of the vitreo-macular interface causing the thickening of the retinal parenchyma at the level of the OPL[33], the presence of fluid in the subretinal space suggests the alteration of the outer BRB being caused by the migration of fluid from the retina through a weakened and permeable ELM or from the hyperpermeable vessels in the choriocapillaris through a dysfunctional RPE[18,32]. In the early stage of the disease the subretinal fluid

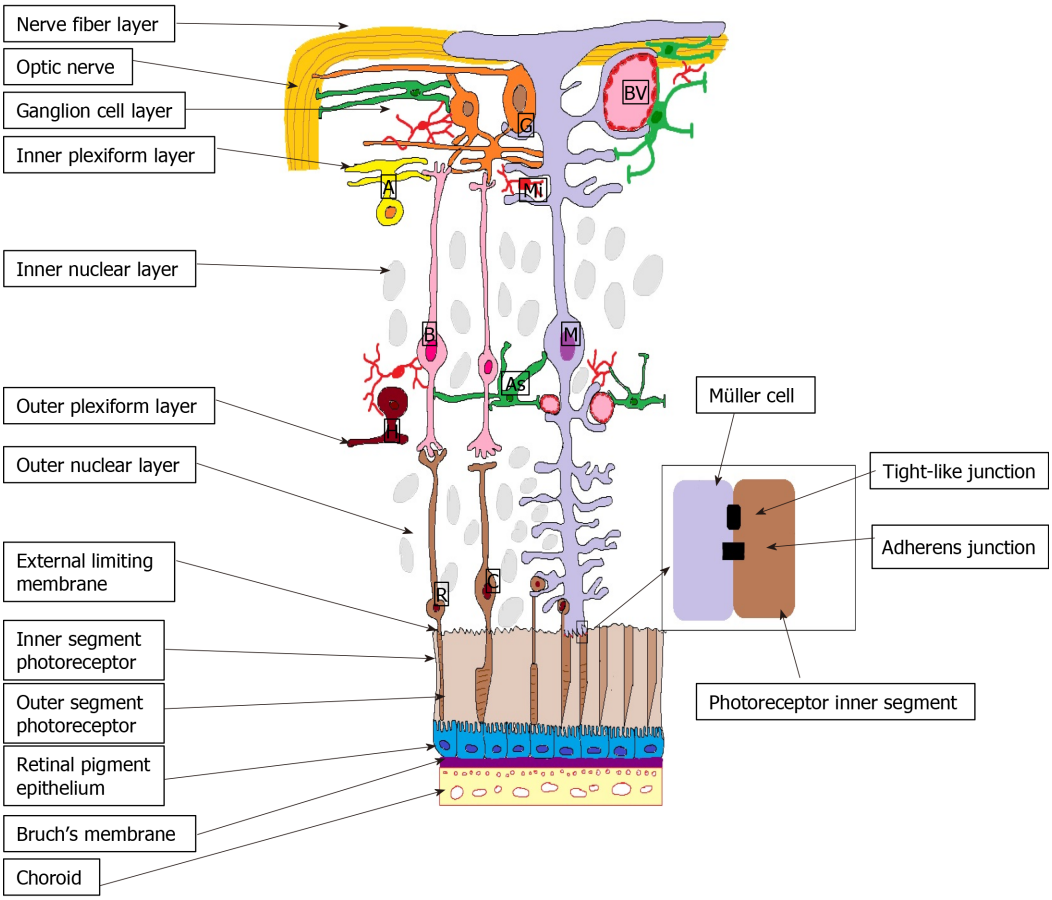

Figure 2 External limiting membrane. M: Müller cells; A: Amacrine cells; G: Ganglion cells; BV: Blood vessels; As: Astrocyte; B: Bipolar cells; R: Rods; C: Cones; H: Horizontal cells; Mi: Microglia; ON: Optic nerve; NFL: Nerve fibre layer; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; ELM: External limiting membrane; IS: Inner segment photoreceptors; OS: Outer segment photoreceptors; RPE: Retinal pigment epithelium; BM: Bruch's Membrane; Ch: Choroid.

originates in the hyperpermeable choriocapillaris through a dysfunctional RPE and as the disease progresses in the breakdown of the outer BRB through a permeable ELM[34,35]. The clinical significance of SRD derives from the observation that its presence is associated with poor visual prognosis, probably due to the disruption of ELM[36].

Several studies compared the effectiveness of anti-VEGF treatment according to the OCT appearance of DME and found that the SRD type which associated ELM and RPE impairment did not respond well[37,38].

Intravitreal triamcinolone was more effective than anti-VEGF therapy in reducing macular thickness and improving vision in eyes with the SRD type of DME (Figure 4) in a prospective case series[39]. However, the relatively short follow-up period (24 mo) requires careful interpretation of these results, especially since long term steroid related complications (cataract, glaucoma) are well known[40,41]. Recently, good results with a dexamethasone implant in SRD were reported and OCT factors associated with better outcomes were identified as the absence of HF and a continuous ellipsoid zone (EZ) at the fovea[42]. The better outcome of SRD following intravitreal steroids as compared to intravitreal anti-VEGF is explained by the role of inflammation in its pathogenesis. In the SRD type of DME increased concentrations of inflammatory cytokines and higher levels of IL-6 were found in the aqueous humor and the vitreous[36]. It is believed that the source of IL-6 is represented by the scavenger cells attracted by the ELM damage[20].

Outer retinal layers

OCT studies offered insights into the outer retina, proving that the disruption of the EZ occurs subsequently to the disruption of ELM[43]. The base of this observation is that ELM has tight junctions between the Müller cells and photoreceptor cells which are similar to those between the RPE cells. As such, ELM is functioning like a third retinal barrier against macromolecules[44] whose malfunctioning leads to the accumulation of

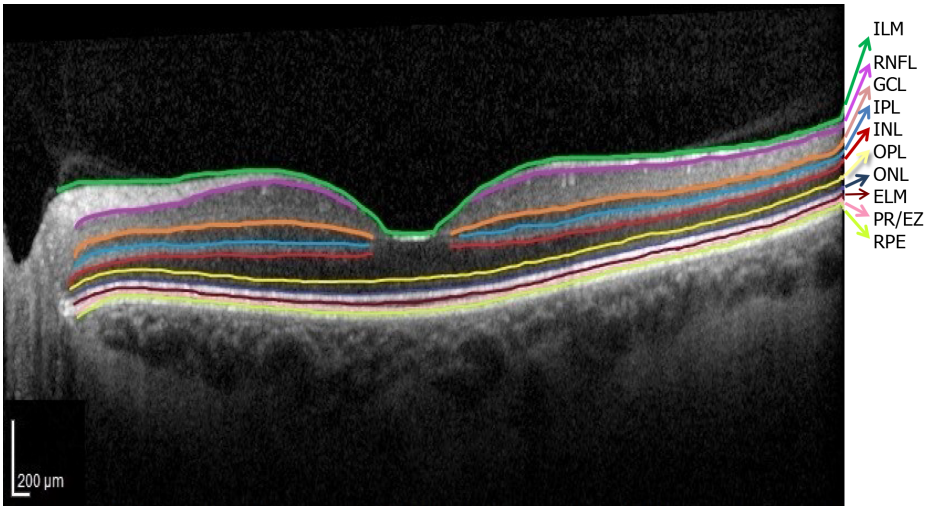

Figure 3 Normal optical coherence tomography aspect of the retinal layers. Segmentation software automatically marked the 10 retinal layers. (ILM: Internal limiting membrane; RNFL: Retinal nerve fiber layer; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; ELM: External limiting membrane; PR/EZ: Photoreceptor layer/ellipsoid zone (inner and outer photoreceptor segment junction; RPE: Retinal pigment epithelium).

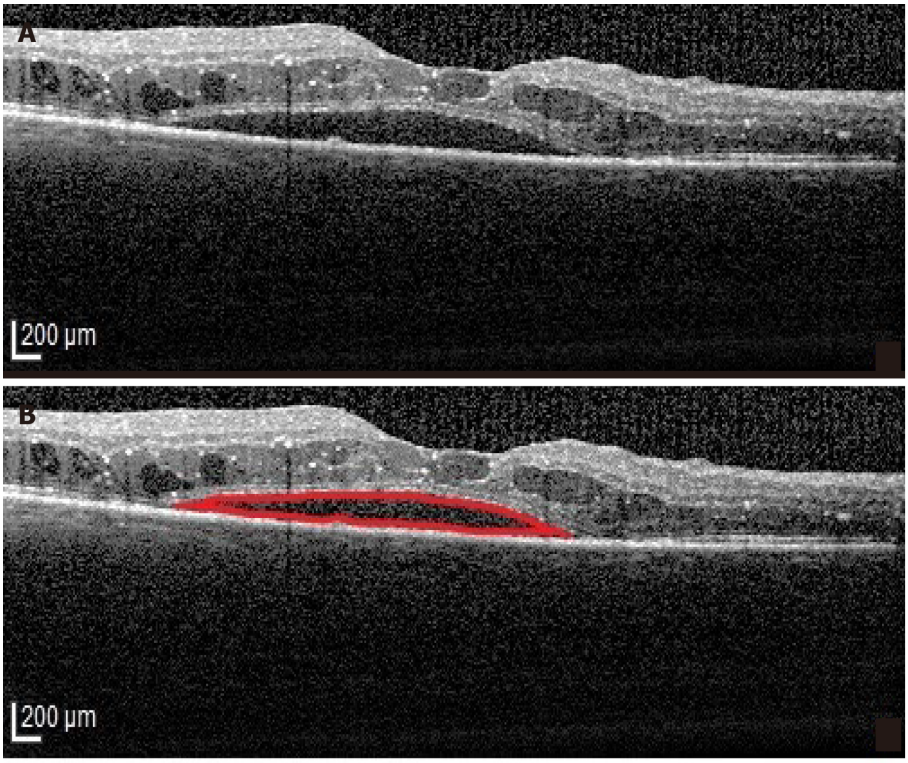

Figure 4 Serous retinal detachment type of diabetic macular edema. A: Optical coherence tomography (OCT)-Retinal neurosensory detachment; B: Highlighted OCT image showing the neurosensory detachment (red).

fluid in DME. As a result, surrogate biomarkers of the outer retina were proposed to determine the progression of DR[45]. A grading of the ultrastructural changes in correlation with the severity of the disease was elaborated, grade 0 meaning no disruption of ELM and EZ, grade 1 meaning ELM disruption with intact EZ and grade 2 meaning disruption of ELM and EZ[46]. The disruption of ELM allows blood components to reach and potentially damage the photoreceptor layer. The damage of ELM in DME could be explained by the extension of the cystoid spaces from the INL to the OPL[47], or by the occurrence of a tear in the outer retinal layers in eyes with SRD[34]. Several studies have shown that ELM disruption is associated with visual impairment in DME[47-49] being a predictor of poor outcomes following the treatment

with triamcinolone[50]. The integrity of ELM and inner segment/outer segment (IS/OS) line was found to be positively correlated with best corrected visual acuity (BCVA) in multiple studies[50-54]. ELM and IS/OS are useful hallmarks for the evaluation of photoreceptor layer whose integrity is closely related to the final BCVA[25].

Murakami *et al*[55] proved that whereas the thickness of the inner retinal layers is positively correlated with the visual impairment, the outer retinal thickness is correlated negatively with poor visual prognosis following vitrectomy for DME. Thus, thinning of the outer retina and photoreceptor degeneration contributes at least partly[33] to the apparently paradoxical changes of visual acuity that were reported by the Diabetic Retinopathy Clinical Research Network[56].

Many studies proved the importance of the inner IS/OS line in DME[47,48,50,51,53,57-60]. It was showed that the transverse length of the disrupted IS/OS line is correlated with the visual acuity[50] and that the length of PROS is associated with the visual function in DME[49]. PROS correlated better with visual acuity than macular thickness, suggesting it as a reliable biomarker of visual acuity in patients with DME[49].

One issue is to point out without a doubt that the IS/OS line that we see on the OCT images corresponds to the actual histological junction between the outer and inner photoreceptors segments. Correlating the microstructures seen on the OCT images with the histological findings, it was speculated that this hyperreflective band was located at the EZ in the inner segments[61]. One important observation is that reflectivity around this band increased after the exposure to light, suggesting that the line is a marker of the photoreceptor function per se[62,63].

Decreased thickness of the PR layer was reported in patients with proliferative diabetic retinopathy-diabetic macular oedema (PDR-DMO) and nonproliferative diabetic retinopathy (NPDR)-DMO[2] and attributed to the reduced values of PROS length in a relatively hypoxic environment at the level of the outer retina[64].

When correlating the OCT parameters of the outer retina with the visual function, Damian *et al*[2] found a low positive correlation between the outer retina and BCVA in the PDR-DME group and a low negative correlation between the RPE thickness and BCVA in the NPDR-DME group. The authors argue that the results are limited by the analyzing of cell thickness not morphology and therefore thickness within normal range is compatible with altered cellular anatomy.

RPE-PR complex

RPE layer is crucial for the survival of PR cells, the two layers being considered as a functional unit due to their interdependence.

In a recent study it was proved that the RPE thickness was decreased in all quadrants in patients with PDR-DME and in some quadrants in the ones with NPDR-DME[2]. This finding may be subsequent to the disruption of the RPE-PR complex possibly due to ischemia[65,66]. Kaarniranta *et al*[67] proved that constant oxidative stress which is a feature of DR leads to the impairment of autophagy and heterophagy in the RPE cells. However, the same authors found occasionally increased RPE thickness in patients with PDR-DME and NPDR-DME[2] which are explained either by the growing of new cells over the RPE cells in order to compensate the fluid leakage within the retina[5] or by the accumulation of shed PROS that are not timely engulfed by the RPE cells due to the alteration of their phagocytosis capacity[68]. The findings of a thickened RPE in diabetic patients may also be a consequence of impaired glycogen metabolism ant its accumulation inside the RPE. It has been shown that glycogen content is increased in the RPE from diabetic donors, as well as in RPE cells grown in hyperglycemic conditions, as consequence of an increase in glycogen synthase activity, whereas the glycogen phosphorylase was normal[69].

Tavares Ferreira *et al*[70] found a thicker RPE layer and thinner PR layer in patients with DM without DR as compared to nondiabetic controls. Xia *et al*[68] reported an increased thickness of the RPE-PR complex measured as a whole, but no changes in the thickness of retinal nerve fiber layer (RNFL) and ganglion cell layer (GCL) in type 2 diabetic patients without retinopathy and concluded that the modifications of the RPE-PR complex preceded the loss of ganglion cells in the diabetic retina without microvascular abnormalities. This finding is consistent with evidence from electrophysiology[71] and color vision which are impaired in patients without clinical DR[72-75].

"Parallelism" of the retinal layers

SD-OCT made it possible to define a new parameter, "parallelism", referring to the integrity of the retinal layers and serving as a potential biomarker to prognosticate visual outcome in DME. The orientation of the photoreceptor status layer at the fovea was categorized including the continuity of ELM, inner segment EZ and presence of

HF in the outer retinal layers. Parallelism was found to be significantly lower in eyes with DME as compared to normal eyes and positively correlated with visual acuity. The absence of HF in the outer retinal layers was associated significantly with higher parallelism and better visual acuity[76].

HF

HF were described as dot like lesions on the OCT images[77] (Figure 5). When identified in the external retinal layers, HF were associated with poor visual outcomes in patients with DME and foveal SRD[34], but also in the ones with DME without SRD[51]. Bolz *et al*[77] postulated that HF in eyes with DME are lipid-laden macrophages and represent the precursors of hard exudates. The observation that the disruption of the ELM and IS/OS line is associated with HF stands for the theory that these lesions reciprocally promote the degeneration of photoreceptor (PR) cells in DME[33].

Correlations between the inner and outer retina

When correlating the inner and outer retinal barriers, Das *et al*[78] found that there is a strong association of disorganization of the inner retinal layers (DRIL) (Figure 6) with the disruption of ELM and EZ in DME, advancing the hypothesis that DRIL could be responsible for the disorganization of the outer retinal architecture. Another study found a highly positive correlation between the thickness of the inner retina and of the central RPE in the NPDR-DME group and a low negative one in the PDR-DME group, stressing the importance of the retinopathy grade on the DME and pointing out that whereas in NPDR the edema involves the entire retina and is mostly vasogenic, in PDR it is driven mainly by ischemia[2].

OPTIAL COHERENCE TOMOGRAPHY (ANGIOGRAPHY) BIOMARKERS OF THE CHOROID IN DME

Enhanced depth imaging OCT and swept source OCT (SS-OCT) allowed to examine the choroid. Vascular changes and thickness alterations of the choroid were reported, outlining the diabetic choroidopathy[79,80].

Choroidal thickness in patients with DM

Several studies have found that DM is associated with decreased choroidal thickness (CT)[80-83]. Since the choroid is the main source of oxygen and nutrients for the outer retina and RPE, this may lead to increased retinal vulnerability to diabetes related hypoxia and ischemia. Moreover, a trend towards choroidal thinning paralleling the increasing severity of DR has been proved[84].

Even in the absence of any clinical retinopathy, some authors reported the significant decrease of CT in patients with DM, suggesting that the decreased choroidal blood flow might be the primary event[80,81] . Choroidal thinning was particularly obvious in the subfoveal and inferior regions in a study conducted by Esmaeelpour *et al*[81]. The same study group noted the perimacular retinal thinning probably caused by the optic nerve fiber layer atrophy[81].

Other studies have shown opposite results, in the sense that thicker choroid was identified in patients with DR[32,80,85]. Tavares Ferreira *et al*[86] measured CT in diabetic patients without DR and found significantly increased CT superiorly to the fovea, proposing it as a possible early preclinical change in diabetes. Using SS-OCT, the same authors identified vascular choroidal remodeling in diabetic patients without retinopathy and choroidal small vessel loss in the areas of previous laser photocoagulation in patients with proliferative diabetic retinopathy[87]. Choroidal thickening increased with the severity of DR, significantly[27] or not significantly[88].

At present there is no consensus on the temporal relationship between the choroidal changes and retinopathy. Some authors claim that the onset of choroidopathy precedes retinopathy, while others argue that the two events are independent[32,80,85,87,89]. Several studies offered evidence of choroidopathy occurring only in the most severe stages of DR[82,83,90] or worsening with the increasing severity of DR[32,89].

CT and DME

Patients with DME have clinically significant thinner subfoveal choroid compared to healthy controls, but when compared to other grades of DR, NPDR and PDR, their choroid is thicker[84]. Kim *et al*[32] reported significantly thicker choroids in DME patients as compared to non-DME patients. When the type of DME was further

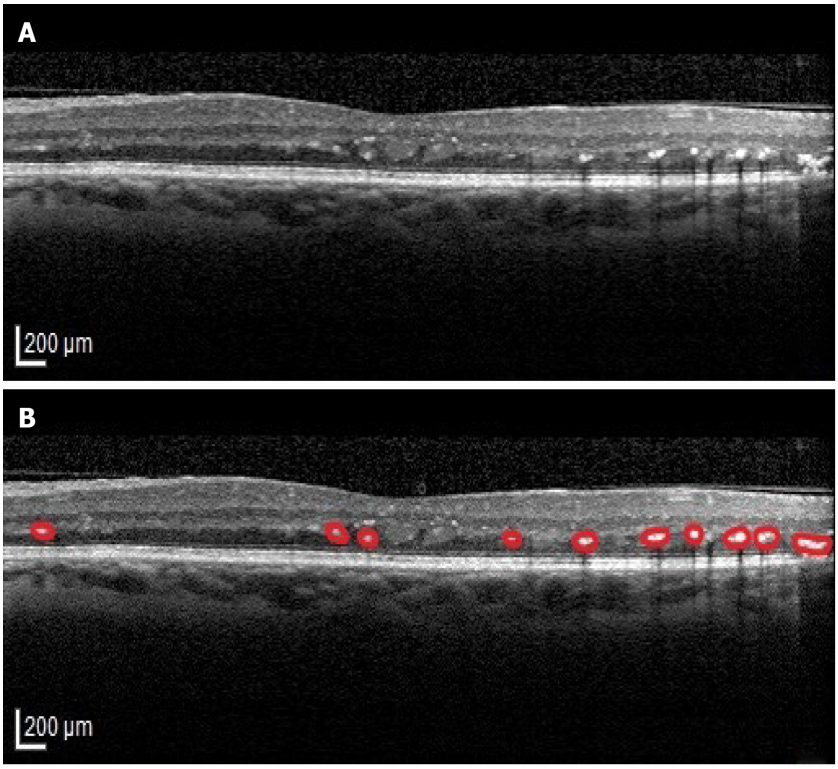

Figure 5 Hyperreflective foci. A: Original optical coherence tomography (OCT) image; B: Highlighted OCT image revealing hyperreflective foci (red).

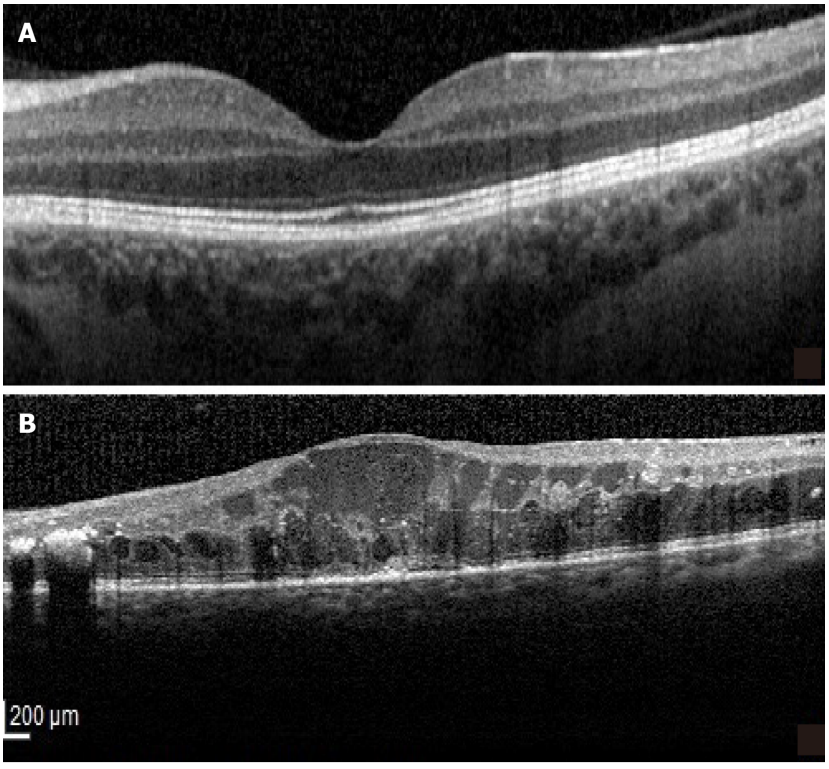

Figure 6 Disorganization of the inner retinal layers. A: Normal optical coherence tomography aspect of the macula; B: Disorganization of the inner retinal layers.

evaluated, CT was significantly greater in the SRD group than in the cystoid type DME[32]. However, other authors reported thinner choroid in case of clinically significant macular edema, explaining this finding as an artifact due to the attenuation of signal transmission and reflection by the edema itself[79,91]. Despite this, Gerendas

et al[91] reported that in the fellow non-affected eye the choroid was also thinner, suggesting that systemic factors are involved in the pathogenesis of this finding. Esmaeelpour *et al*[81] reported no choroidal thinning below the lesions in patients with DME.

CT after treatment for DR

It was shown that panretinal photocoagulation (PRP) is followed initially by choroidal swelling within one week, which is explained by the shifting of vessels from the peripheral retina to the foveal area[92,93], followed by the thinning of the choroid, possibly by downregulation of VEGF[27]. Cho *et al*[93] found at 1 and 3 mo after PRP an increased subfoveal CT concomitant with a significant CT decrease in the photocoagulated area.

Regarding anti-VEGF treatment, several studies reported choroidal thinning over the first 6 mo[94,95]. Rayess *et al*[96] showed that subfoveal choroidal thickness (SFCT) is a predictor of response to anti-VEGF therapy in the sense that a greater SFCT at baseline is associated with better outcomes. One explanation is that greater choroidal thickness stands for intact choriocapillaris, less ischemic outer retina and better preservation of photoreceptors[96].

Systemic factors, like blood hemoglobin, arterial blood pressure and hypercholesterolemia, influence CT[84].

Future developments of CT as biomarker in DME

CT in patients with DR is a highly unreliable parameter and multiple studies show different results because there is a poor control of variables, a wide range of collecting data and different devices are used. There is evidence that the choroid thins with progressing DME as well as following PRP and anti-VEGF injections. It was reported that longer standing DME is associated with worse anatomical and functional outcomes following anti-VEGF treatments. Therefore, a thicker choroid prior to treatment would probably indicate a shorter duration DME and be associated with better outcomes after treatment. Thus, CT may be also attributed the role of prognostic biomarker able to predict the response to treatment in DME. However, in order to get reliable data on CT, future studies should accomplish certain requirements: to define clearly the scleral-choroidal junction, to include local (refractive status), and general (age, diabetes duration, HbA1C) factors in the analysis, to make a longitudinal approach and use longer follow up intervals[28].

Choroidal vascular index in patients with DM

Choroidal vascular index (CVI) is a term that means the ratio of choroidal luminal area to total choroidal area which was recently introduced as a novel biomarker to monitor the progression of DR[97]. CVI may be attributed the role of an early biomarker, because studies proved that while CT is unaltered in DR, CVI correlates with progressing DR[83]. CVI alteration before the onset of DR, supports the theory of choroidal primary damage in DR[98].

Decreased choroidal blood flow creates a hypoxic environment for the RPE and photoreceptor cells, disrupting the phagocytosis and rendering the RPE cells fragile[99,100]. The condition is aggravated by the subsequent production of superoxide and soluble inflammatory factors[68].

Whereas most of the studies focused on the CT showing its thinning in patients with DM, proportional with the severity of DR, a multicenter cross-sectional study used SS-OCT images to analyze choroidal vascularity in different stages of DR and introduced new quantitative parameters, such as choroidal vascular density (CVD) and choroidal vascular volume (CVV)[28]. According to this study, the eyes with DME and PDR had a reduced CVD and eyes with PDR had also a reduced CVV compared to controls, reflecting the notion that vascular abnormalities increase with the severity of DR. In eyes with NPDR without DME, the overall CVD was significantly reduced, but not at macula, suggesting that although diffuse choroidopathy may be present in early stages of DR, submacular choroidopathy only becomes present in later stages of DR. The same authors proved that in diabetic patients without DR, the choroidal vascular indices did not show significant differences compared to controls. Thus, it is suggested that the occurrence of diabetic choroidopathy does not precede that of retinopathy, although further studies are required to elucidate this important issue for understanding the diabetic eye disease[28].

A recent study analyzed CVI after intravitreal injection of ranibizumab in eyes with DME and found the significant reduction of CVI and choroidal blood flow only in the no-PRP group, but not in the PRP-treated group. Moreover, a significant correlation

between the central retinal thickness and choroidal blood flow was found in the no-PRP group[101].

OCTA choroidal biomarkers in DME

In diabetic patients without DR, an OCTA study showed that the choroidal foveal flow area was significantly decreased compared to controls suggesting that the compromise of the circulation starts in the foveal choroidal layer in DR, preceding the occurrence of mycroaneurysms. When DR develops, both the retinal and choroidal capillaries are significantly reduced[102].

INDOCYANINE GREEN ANGIOGRAPHY

Years before the recent studies with OCT, a paper using indocyanine green angiography (ICGA) in patients with NPDR disclosed microvascular findings in addition to those described with fluorescein angiography: lobular spotty hyperfluorescent and hypofluorescent areas in the very late phase, diffuse late-phase hyperfluorescence corresponding to retinal capillary non-perfusion areas on fluorescein angiography and retinal edema[103]. However, due to its invasiveness and lack of quantification, ICGA is limited in detecting ischemia in early DR[102].

CONCLUSION

Advances in OCT technology allow a more detailed investigation of the outer retina and choroid providing biomarkers that mediate the decoding of pathogenesis, monitoring and selection of the best treatment option for DME. By identifying new OCT biomarkers at the level of outer retina and choroid, paths for early diagnosis and identification of novel therapeutic targets in DME are opened.

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ORIGINAL ARTICLE

Basic Study

Effect of oligofructose on resistance to postoperative high-fat diet-induced damage of metabolism in diabetic rats after sleeve gastrectomy

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Institutional animal care and use

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Abstract

BACKGROUND

Sleeve gastrectomy (SG) can induce prominent remission of type 2 diabetes mellitus. However, the long-term remission rate of diabetes usually decreases over time. Oligofructose has been verified to modulate host metabolism. The aim of this study was to explore the protective effect of oligofructose on high-fat diet (HFD)-induced metabolic dysfunction after SG.

AIM

To study the effect and mechanism of oligofructose on diabetic remission in diabetic rats after SG.

METHODS

SG and SHAM operation were performed on diabetes rats induced with an HFD, nicotinamide, and low-dose streptozotocin. Then the rats in the SHAM and SG

committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care Committee of the First Affiliated Hospital of Shandong First Medical University, Jinan, China (Protocol No. 2018-006).

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groups were continuously provided with the HFD, and the rats in sleeve gastrectomy-oligofructose group were provided with a specific HFD containing 10% oligofructose. Body weight, calorie intake, oral glucose tolerance test, homeostasis model assessment of insulin resistance, lipid profile, serum insulin, glucagon-like peptide 1 (GLP-1), total bile acids, lipopolysaccharide (LPS), and colonic microbiota levels were determined and compared at the designated time points. All statistical analyses were performed using Statistic Package for Social Science version 19.0 (IBM, United States), and the statistically significant difference was considered at $P < 0.05$.

RESULTS

At 2 wk after surgery, rats that underwent SG exhibited improved indexes of glucose and lipid metabolism. Compared with the SG group, the rats from SG-oligofructose group exhibited better parameters of glucose and lipid metabolism, lower body weight (526.86 ± 21.51 *vs* 469.25 ± 21.84, $P < 0.001$), calorie intake (152.14 ± 9.48 *vs* 129.63 ± 8.99, $P < 0.001$), homeostasis model assessment of insulin resistance (4.32 ± 0.57 *vs* 3.46 ± 0.52, $P < 0.05$), and LPS levels (0.19 ± 0.01 *vs* 0.16 ± 0.01, $P < 0.05$), and higher levels of insulin (1.17 ± 0.17 *vs* 1.58 ± 0.16, $P < 0.001$) and GLP-1 (12.39 ± 1.67 *vs* 14.94 ± 1.86, $P < 0.001$), and relative abundances of *Bifidobacterium* (0.0034 ± 0.0014 *vs* 0.0343 ± 0.0064, $P < 0.001$), *Lactobacillus* (0.0161 ± 0.0037 *vs* 0.0357 ± 0.0047, $P < 0.001$), and *Akkermansia muciniphila* (0.0050 ± 0.0024 *vs* 0.0507 ± 0.0100, $P < 0.001$) at the end of the study. However, no difference in total bile acids levels was observed between the two groups.

CONCLUSION

Oligofructose partially prevents HFD-induced glucose and lipid metabolism damage after SG, which may be due to the changes of calorie intake, insulin, GLP-1, LPS, and the gut microbiota in rats.

Key Words: Sleeve gastrectomy; Oligofructose; Diabetes; Gut microbiota; Lipopolysaccharide; Glucagon-like peptide 1

Core Tip: Bariatric surgery is one of the important methods to treat obesity and type 2 diabetes mellitus, but the remission and recurrence of type 2 diabetes mellitus after surgery are still a hot issue. In this study, we demonstrated that oligofructose can partially resist the high-fat diet-induced glucose and lipid metabolism damage after sleeve gastrectomy in rats, and the influence of oligofructose on calorie intake, insulin, glucagon-like peptide-1, lipopolysaccharide, and gut microbiota may play an important role in the improving function.

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INTRODUCTION

Bariatric surgery has been considered the most effective treatment for type 2 diabetes mellitus (T2DM)[1,2]. Over the past decade, the total number of bariatric surgeries worldwide is increasing, particularly sleeve gastrectomy (SG). Currently, SG is the most frequently used procedure in the United States/Canada and in Asian/Pacific regions[3]. Although bariatric surgery induces rapid and prominent remission of T2DM, the long-term remission rate usually decreases over time, and the recurrence of T2DM is observed in a part of patients with initial remission after bariatric surgery[4-7]. As a novel type of bariatric surgery, SG is characterized by lower complication rate,

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faster operation, fewer technical requirements, and fewer postoperative nutritional problems[8], so that a marked increase in the relative application rate of SG has been observed[3]. Many investigators have reported that SG and Roux-en-Y gastric bypass (RYGB) surgery have equal treatment efficacy for diabetes[9,10]. However, long-term randomized controlled comparison for the impact of SG coupled with RYGB as the gold standard in subjects with T2DM is surprisingly limited. The long-term effect of SG on diabetes is questionable[8]. What is more, SG has been reported to be an independent predictor of the relapse of diabetes at 48.7 mo of follow-up[5]. How to reduce or delay the recurrence of diabetes after surgery is a severe problem that the clinicians have to face.

Prebiotics are non-digestible oligosaccharides, such as oligofructose, galactooligosaccharides, lactulose, and inulin. Prebiotics promote the loss of body weights and improve the metabolism of glucose and lipid in rodents and human[11-15]. The mechanisms underlying these benefits may be due to the reduction in energy intake, regulation of gut microbiota, improvements in low-grade inflammation, and increased levels of gut hormones, such as glucagon-like peptide-1 (GLP-1) and peptide YY[16-19], which prompted us to explore whether prebiotics reduce or delay the recurrence of diabetes after surgery.

A high-calorie diet appears to be one of primary factors contributing to obesity and diabetes[20], and our previous studies have confirmed that a high-fat diet (HFD) induces the deterioration of glucose tolerance after an initial improvement in diabetic rats subjected to duodenal-jejunal bypass[21]. In the present study, we conducted SG on nicotinamide-streptozotocin (STZ)-HFD-induced diabetic model rats with metabolic characteristics of human diabetes[22-24], and stimulated the recurrence of diabetes with postoperative HFD feeding. The glucose and lipid profiles, serum levels of insulin, GLP-1, total bile acids (TBA), and lipopolysaccharide (LPS), and changes in the composition of the gut microbiota were determined and compared.

MATERIALS AND METHODS

Animals and diets

Forty 8-wk-old male Wistar rats (Laboratory Animal Center of Shandong University, Jinan, China) were individually housed in independent ventilated cages at a constant temperature (24-26 °C), humidity (50%-60%), and a light-dark cycle (12 h light: 12 h dark). All animal procedures were approved by the Institutional Animal Care Committee of the First Affiliated Hospital of Shandong First Medical University. All rats were provided with an HFD (40% of calories as fat, Huafukang Biotech Company, Beijing, China) for 4 wk to induce insulin resistance, and then treated with a single intraperitoneal injection of nicotinamide (170 mg/kg, Sigma, St. Louis, MO, United States) followed by a single injection of STZ (65 mg/kg, Sigma, St. Louis, MO, United States) 15 min later after 12 h of fasting to induce a diabetic state. Two weeks after STZ injection, 29 rats were selected as diabetic rats with a fasting blood glucose level higher than 7.1 mmol/L or a blood glucose level after gavage for 2 h higher than 11.1 mmol/L during the oral glucose tolerance test (OGTT), and the rats with extreme hyperglycemia (blood glucose level > 16.7 mmol/L) were excluded from the study[22-24]. The diabetic rats were randomly allocated to a SHAM group (n = 9), SG group (n = 10), and SG-oligofructose (SG-OF) group (n = 10). The rats in the SHAM and SG groups were continuously provided with the HFD after the operation, and the rats in the SG-OF group were provided with a specific HFD supplemented with 10% oligofructose (Huafukang Biotech Company, Beijing, China) since 2 wk after surgery. Body weight and calorie intake were measured at baseline, and 2, 12, and 24 wk after surgery.

Surgical techniques

The diabetic rats were fed a low-residue diet from 48 h before the operation to 72 h after the operation. Then, they were allowed for *ad libitum* access to the HFD and water. Rats were anesthetized with a 10% chloral hydrate solution during surgery. The surgical processes were performed as described in our previous study and a published report[25,26].

SG surgery: The surgical procedure is as follows: (1) A 4 cm midline abdominal incision was made from the xiphoid process; (2) The gastric omentum was dissected to disclose the gastric cardium; (3) Short gastric vessels, corresponding gastroepiploic vessels, and the branches of left gastric vessels in the greater curvature were ligated

and transected using a 7-0 silk suture (Ningbo Medical Needle, Ningbo, Zhejiang, China); (4) A vena caval clamp was placed to occlude the stomach wall through the cardia to pylorus for the prevention of bleeding; (5) The gastric fundus and a large portion of gastric body were removed (70% of the total stomach); and (6) The residual stomach was closed using a 7-0 silk suture (Ningbo Medical Needle, Ningbo, Zhejiang Province, China).

SHAM surgery: A similar abdominal incision was made as described for the SG procedure, and a similar process was conducted, but the gastric fundus and the gastric body were not removed. The operation time of the SHAM surgery was prolonged to the same time as SG surgery to acquire similar surgical and anesthetic stress.

OGTT

OGTT was performed at baseline and 2, 12, and 24 wk after surgery. After 8 h of fasting, all rats were subjected to gavage with glucose at a dose of 1 g/kg. Blood glucose levels were measured in conscious rats at baseline and 10, 30, 60, 90, and 120 min after gavage.

Serum parameters

During the OGTT conducted at baseline and 2, 12, and 24 wk after surgery, blood samples were collected from the retrobulbar venous plexus at baseline and 10, 30, 60, and 120 min after glucose gavage. Blood samples were centrifuged (1000 × *g*) at 4 °C for 15 min, and serum was immediately extracted and stored at -80 °C. Fasting serum TBA levels and serum lipid profiles were measured using an automatic biochemical analyzer (Hitachi, Tokyo, Japan). Serum insulin and fasting serum GLP-1 and LPS levels were measured using enzyme-linked immuno-sorbent assay kits (insulin: Millipore, MA, United States; GLP-1: Uscn Life Science incorporated, Wuhan, Hubei, China; LPS: Bio-Swamp, Wuhan, Hubei Province, China).

Homeostasis model assessment of insulin resistance

At baseline and 2, 12, and 24 wk after the operation, homeostasis model assessment of insulin resistance (HOMA-IR) was calculated to evaluate insulin resistance using the following formula: HOMA-IR = fasting insulin (mIU/L) × fasting glucose (mmol/L)/22.5[27].

16S rDNA sequence analysis of gut microbiota

The change in the gut microbiota may alter host metabolism partially due to the biological activity of the colonic microbiota. Thus, the colonic contents were collected at 24 wk after surgery, and stored at -80 °C immediately. Genomic DNA was extracted from the colonic contents using standard methods[28]. Amplicons of the 16S rRNA gene V4 region were sequenced using the Illumina MiSeq platform (BGI technology, Shenzhen, Guangdong Province, China).

Statistical analysis

All data are presented as the mean ± SD. Areas under the curves for OGTT (AUC_{OGTT}) were calculated using the trapezoidal integration. The data in each group were analyzed by Shapiro-Wilk test for normality. Intergroup comparisons were evaluated using one-way analysis of variance followed by Bonferroni post hoc comparison. The concentrations of insulin and total GLP-1 after glucose gavage were analyzed using a mixed model analysis of variance followed by Bonferroni post hoc comparison. All statistical analyses were performed using Statistic Package for Social Science version 19.0 (IBM, United States), and the statistically significant difference was considered at $P < 0.05$.

RESULTS

Oligofructose promotes the improvement of insulin resistance after SG in rats

At the end of this experiment, 9, 7, and 8 rats were alive in the SHAM, SG, and SG-OF groups, respectively. Five rats died of residual stomach leakage after SG. As shown in Figure 1A, the AUC_{OGTT} of the three groups were not significantly different at baseline assessment. At 2, 12, and 24 wk after surgery, the AUC_{OGTT} in the SG and SG-OF groups were both lower than those in the SHAM group. The difference of AUC_{OGTT} between the SG group and SG-OF group was observed since 24 wk after surgery. At 24

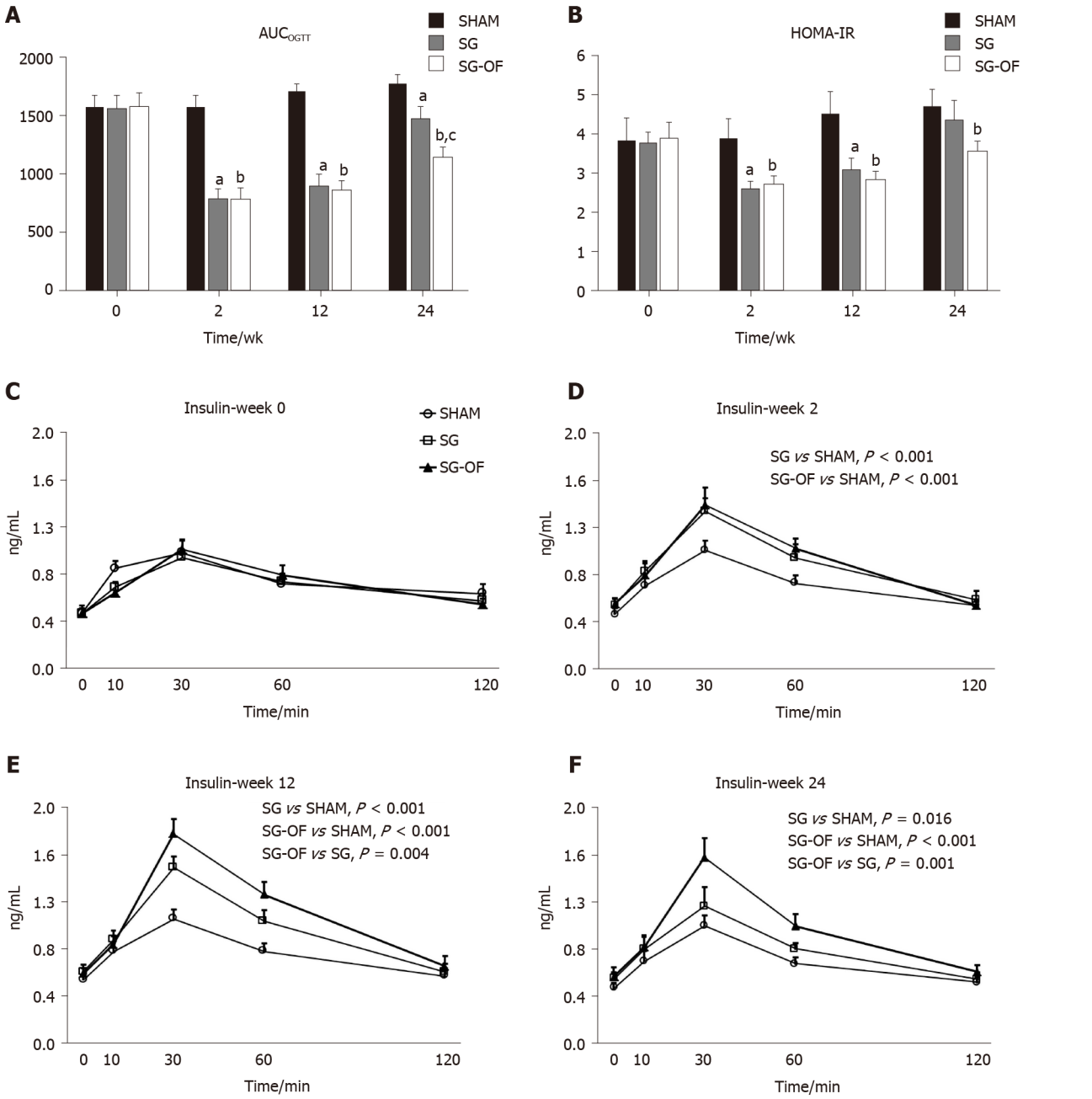

Figure 1 Areas under the curves for oral glucose tolerance test, homeostasis model assessment of insulin resistance, and serum insulin levels. A: Areas under the curves for oral glucose tolerance test; B: Homeostasis model assessment of insulin resistance; C-F: Serum insulin levels measured during the oral glucose tolerance test at baseline and 2, 12, and 24 wk after surgery [$^{a}P < 0.001$, SHAM *vs* sleeve gastrectomy (SG); $^{b}P < 0.001$, SHAM *vs* SG-oligofructose (SG-OF); $^{c}P < 0.001$, SG *vs* SG-OF]. The concentration of insulin during the oral glucose tolerance test was analyzed using mixed model ANOVA followed by Bonferroni post hoc comparison. The *P* value is shown in the rectangular frame. A statistically significant difference was considered at $P < 0.05$. AUC$_{OGTT}$: Areas under the curves for oral glucose tolerance test; SG-OF: Sleeve gastrectomy-oligofructose; HOMA-IR: Homeostasis model assessment of insulin resistance.

wk after surgery, rats from the SG-OF group showed significantly lower AUC$_{OGTT}$ than rats from the SG group.

HOMA-IR showed a similar trend to the AUC$_{OGTT}$, as shown in Figure 1B. No significant differences of HOMA-IR among the three groups were observed at baseline assessment. At 2 and 12 wk after surgery, the rats that underwent SG showed significantly lower HOMA-IR than that from the SHAM group. At 12 wk after surgery, the HOMA-IR of the SG group appeared to be higher than that in the SG-OF group, but there was no statistic difference between the two groups. At 24 wk after surgery, the HOMA-IRs in the SHAM and SG groups were comparable, and higher than that in the SG-OF group.

The curves of serum insulin levels measured during OGTT at baseline and 2, 12, and 24 wk after surgery are shown in Figure 1C-F, respectively. No significant difference of serum insulin levels was observed among the three groups at baseline assessment. A

greater amount of insulin was secreted into the serum in the SG and SG-OF groups during the OGTT than that in the SHAM group at 2, 12, and 24 wk after surgery. A difference in the secretion of insulin into the serum was observed between the SG and SG-OF groups, and detected earlier than changes in the AUC_{OGTT} and HOMA-IR. A lower amount of insulin was secreted into the serum of the SG group than the SG-OF group beginning at 12 wk after surgery.

Oral oligofructose can improve postoperative lipid profiles

Fasting serum triglyceride and cholesterol levels are shown at Figure 2. There was no significant difference in lipid profiles among the three groups at baseline assessment. And triglyceride and cholesterol in the SG and SG-OF groups were significantly lower than those in the SHAM group at 2 and 12 wk after surgery. At 12 wk after surgery, the SG-OF group showed a lower triglyceride level than the SG group and a comparable cholesterol level to the SG group. At 24 wk after surgery, the SHAM group showed the highest triglyceride level among the three groups and the triglyceride level in the SG group was higher than that in the SG-OF group. Cholesterol levels in the SG group were comparable to the levels in the SHAM group, but higher than those in the SG-OF group.

The mechanism of diabetes remission may be related to weight loss and energy intake

As shown in Figure 3, body weight and calorie intake exhibited a similar trend during this experiment. No significant differences in body weight or calorie intake were observed among the three groups at baseline, and all rats subjected to SG showed a lower body weight and calorie intake than the rats from the SHAM group at 2, 12, and 24 wk after surgery. The body weight and calorie intake in the SG-OF group were lower than those in the SG group beginning at 12 wk after surgery.

Oligofructose can increase GLP-1 level and lower LPS level, but has limited effect on TBA

The fasting serum GLP-1 levels at baseline and 2, 12, and 24 wk after surgery are shown in Figure 4A-D, respectively. All the three groups showed similar baseline levels of GLP-1, but the SG and SG-OF groups showed higher GLP-1 levels at all postoperative time points. Compared with the SG group, the SG-OF group exhibited higher GLP-1 levels beginning at 12 wk after surgery.

As shown in Figure 4E, there was no significant difference in LPS levels between groups at baseline assessment. In contrast to the GLP-1 levels, the SG and SG-OF groups showed lower LPS levels at all postoperative time points than the SHAM group, and the LPS level in the SG-OF group was lower than that in the SG group beginning at 12 wk after surgery.

The fasting serum TBA levels are shown in Figure 4F. At all postoperative time points, the TBA levels in the SG and SG-OF groups were significantly higher than those in the SHAM group, but no significant difference was observed between the SG group and SG-OF group.

Oligofructose can change gut microbiota

As shown in Figure 5A and B, *Bacteroidetes* were the predominant gut microbes in the SHAM group while *firmicutes* were dominant in the SG and SG-OF groups. The relative abundance of *Bifidobacterium* (Figure 5C), *Lactobacillus* (Figure 5D), and *Akkermansia muciniphila* (Figure 5E) in the SG-OF group was significantly higher than that in the SHAM and SG groups. There was no difference in relative abundance of *Bifidobacterium* and *Akkermansia muciniphila* between the SHAM group and SG group. The SG group showed a significantly higher relative abundance of *lactobacillus* than the SHAM group.

DISCUSSION

Compared with nonsurgical treatment, bariatric surgery has achieved a higher remission rate of T2DM[2,29]. In addition, SG is characterized by faster operation, fewer technical requirements, lower complication rate, and fewer postoperative nutritional problems[8]. In recent years, SG has been performed more frequently than RYGB in the United States/Canada and in Asian/Pacific regions[3]. However, as a novel surgical approach, the long-term effect of SG on diabetes is questionable. Suboptimal loss and

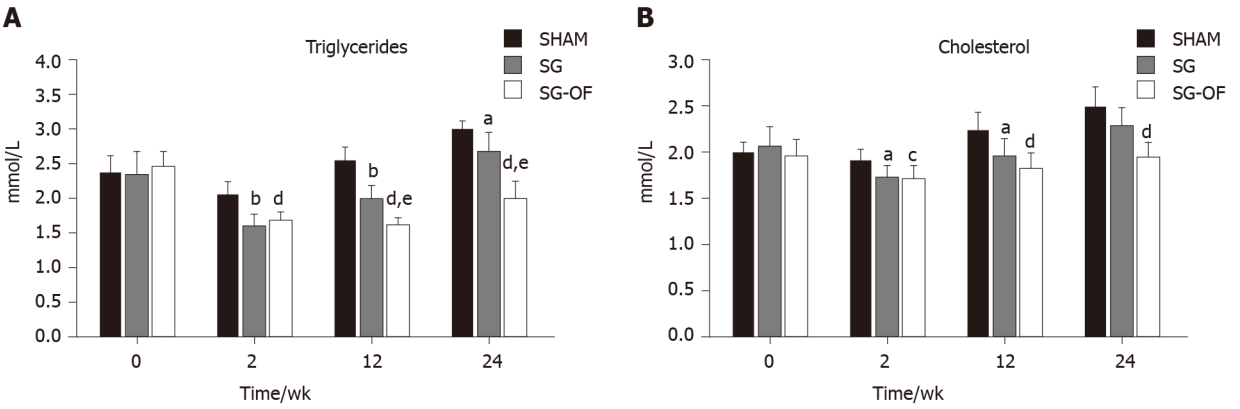

Figure 2 Fasting serum triglyceride and cholesterol levels. A: Fasting serum triglyceride levels; B: Fasting serum cholesterol levels [$^{a}P < 0.05$, $^{b}P < 0.001$, SHAM *vs* sleeve gastrectomy (SG); $^{c}P < 0.05$, $^{d}P < 0.001$, SHAM *vs* SG-oligofructose (SG-OF); $^{e}P < 0.001$, SG *vs* SG-OF]. A statistically significant difference was considered at $P < 0.05$. SG-OF: Sleeve gastrectomy-oligofructose.

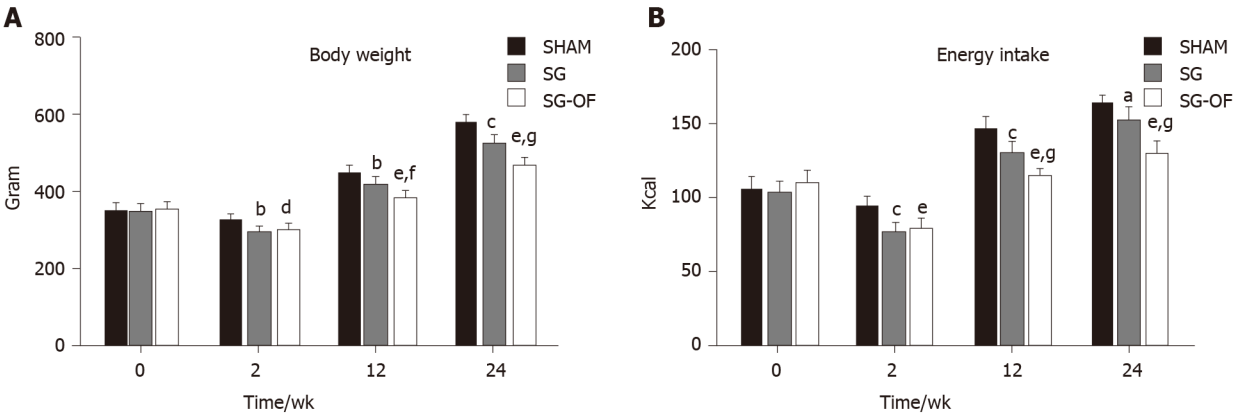

Figure 3 Body weight and calorie intake. A: Body weight measured at baseline and 2, 12, and 24 wk after surgery; B: Calorie intake measured at baseline and 2, 12, and 24 wk after surgery [$^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, SHAM *vs* sleeve gastrectomy (SG); $^{d}P < 0.01$, $^{e}P < 0.001$, SHAM *vs* SG-oligofructose (SG-OF); $^{f}P < 0.01$, $^{g}P < 0.001$, SG *vs* SG-OF]. A statistically significant difference was considered at $P < 0.05$. SG-OF: Sleeve gastrectomy-oligofructose.

regain of body weight are associated with noncompliant dietary and lifestyle habits[30]. Suboptimal loss and regain of body weight have been demonstrated as the factors for insufficient control of diabetes following bariatric surgery[31,32]. Prebiotics have been considered as an effective management tool to promote body weight loss and improve metabolism of glucose and lipid[11-14]. As shown in our previous study, HFD reverses the improvements in diabetes after bariatric surgery[21]. In the present study, we conducted SG on diabetic rats, and stimulated the reversion of improvements in diabetes with sustained postoperative HFD feeding. Meanwhile, a portion of the SG rats were provided with a specific HFD supplemented with 10% oligofructose (one kind of prebiotic) to explore whether oligofructose prevents the reversion of improvements in diabetes after SG.

In this study, at 2 wk after surgery, the AUC_{OGTT} of the SG rats was significantly lower than that of the SHAM rats. Following postoperative HFD feeding, the AUC_{OGTT} of the SG rats showed a gradually increasing trend. At 24 wk after surgery, the AUC_{OGTT} of the SG group was significantly higher than that of the SG-OF group. The changing trends of insulin, HOMA-IR, triglyceride, and cholesterol levels were similar to that of the AUC_{OGTT}. Among these factors, the difference in insulin secretion during the OGTT and triglyceride levels (at 12 wk after surgery) revealed an earlier appearance than others (at 24 wk after surgery), suggesting that, at the early stage after surgery, SG improves the metabolism of glucose and lipid obviously; postoperative HFD feeding reverses these metabolic improvements, and oligofructose feeding partially prevents the detrimental effect of HFD feeding. Factors such as body weight, calorie intake, GLP-1 levels, serum TBA levels, LPS levels, and the gut microbiota were measured to elucidate the possible mechanisms underlying the protective effect of oligofructose.

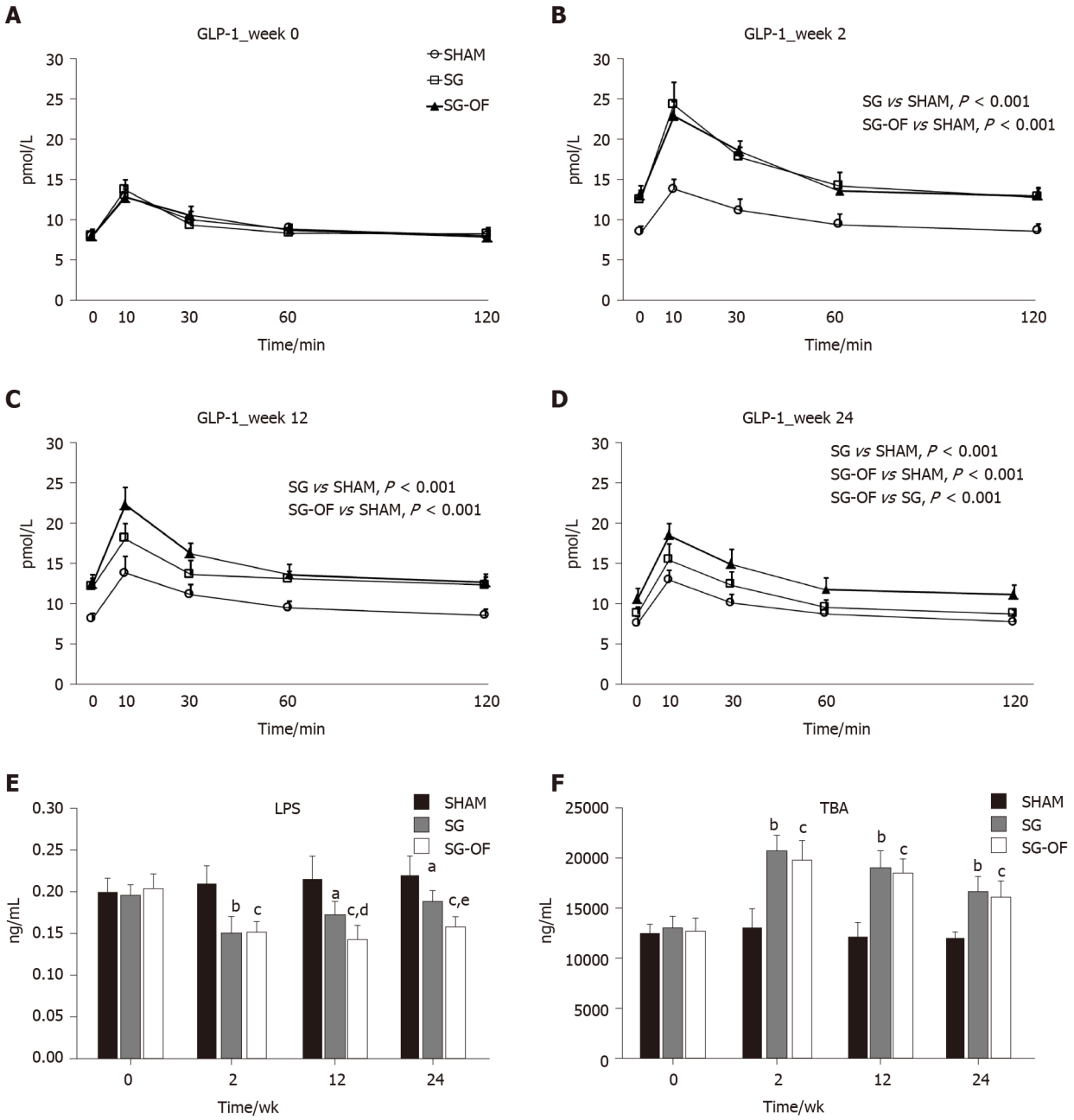

Figure 4 Glucagon-like peptide 1, fasting serum lipopolysaccharide, and total bile acids levels. A-D: Fasting serum glucagon-like peptide 1 levels measured at baseline and 2, 12, and 24 wk after surgery. The *P* value is shown in a rectangular frame; E: Fasting serum lipopolysaccharide; F: Total bile acids [$^{a}P < 0.01$, $^{b}P < 0.001$, SHAM *vs* sleeve gastrectomy (SG); $^{c}P < 0.001$, SHAM *vs* SG-oligofructose (SG-OF); $^{d}P < 0.05$, $^{e}P < 0.01$, SG *vs* SG-OF]. A statistically significant difference was considered at $P < 0.05$. GLP-1: Glucagon-like peptide 1; LPS: Lipopolysaccharide; TBA: Total bile acids; SG-OF: Sleeve gastrectomy-oligofructose.

The body weight and calorie intake in the SG group were obviously lower than those in the SHAM group at all postoperative time points, and a lower body weight and calorie intake were observed in the SG-OF group but not in the SG group beginning at 12 wk after surgery. Body weight and calorie intake seem to be one of primary factors contributing to diabetes[20], so we hold the opinion that lower body weight and calorie intake may be one of the mechanisms for the protective effect of SG and oligofructose.

As one of the most important incretin hormones, GLP-1 is secreted by L-cells that are mainly located in the epithelium of the distal ileum and colon[33]. It regulates glucose homeostasis by stimulating insulin secretion, suppressing glucagon secretion, and promoting proliferation and inhibiting apoptosis of β cells[34]. In our study, a greater amount of GLP-1 was secreted in rats subjected to SG during the OGTT than in rats in the SHAM group at all postoperative time points, and a higher level of GLP-1 secretion in the SG-OF group than in the SG group was observed beginning at 12 wk after surgery. GLP-1 has been considered a mediator in the remission of diabetes after

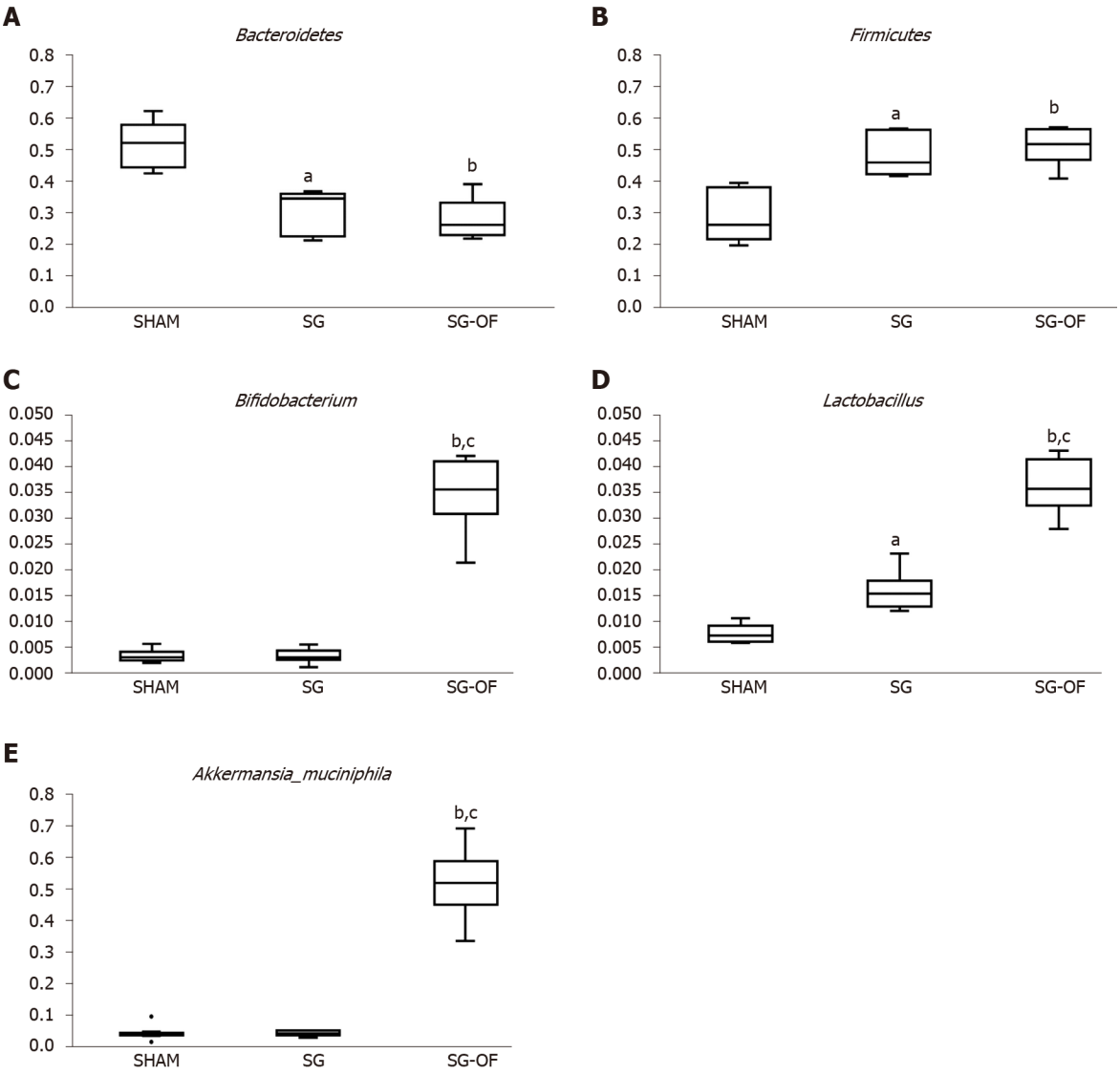

Figure 5 Gut microbiota. A: *Bacteroidetes*; B: *Firmicutes*; C: *Bifidobacterium*; D: *Lactobacillus;* E: *Akkermansia_muciniphila.* The relative abundances of *Bacteroidetes*, *Firmicutes*, *Bifidobacterium*, *Lactobacillus*, and *Akkermansia_muciniphila* between groups were analyzed using the ANOVA and a statistically significant difference was considered at $P < 0.05$ ($^{a}P < 0.001$, SHAM *vs* sleeve gastrectomy (SG); $^{b}P < 0.001$, SHAM *vs* SG-oligofructose (SG-OF); $^{c}P < 0.001$, SG *vs* SG-OF). SG-OF: Sleeve gastrectomy-oligofructose.

SG[35], and oligofructose has been demonstrated as the promoter for the production of endogenous GLP-1[36]. Based on these findings, we have deduced that the function of oligofructose for improving glucose metabolism after SG is partially attributed to the enhanced secretion of GLP-1. The mechanisms for the enhanced secretion of GLP-1 after oligofructose feeding may be due to the increased number of L-cells and colonic fermentation[36,37]. Short-chain fatty acids [(SCFAs); butyrate, propionate, and acetate)] are produced through the fermentation of nondigestible carbohydrates in the colon, and it has been reported that the oral administration of butyrate and propionate in mice can significantly increase plasma levels of GLP-1, thus leading to an improvement in insulin sensitivity[38], which is consistent with another report showing that SCFAs stimulate free fatty acid receptor 2 that is colocalized in L-cells to increase the secretion of GLP-1[39]. Unfortunately, SCFAs were not determined in our study.

Bile acids can act as signaling molecules to regulate the metabolism of lipid, glucose, and energy, and these regulatory functions of bile acids are predominantly mediated by farnesoid X receptor and the G-protein-coupled receptor TGR5[40]. In this study, at 2 wk after surgery, fasting serum TBA of the rats subjected to SG was significantly higher than that in the SHAM group. This result is coincident with the previous report in human[41], showing that the increased TBA levels contribute to the improvements in the metabolism of glucose and lipid after SG. However, we did not observe a significant difference in TBA levels between the SG group and SG-OF group at 12 and 24 wk after surgery, indicating that the protective effect of oligofructose may be

independent of the increased TBA levels.

LPS is a component of the cell wall in Gram-negative intestinal microbiota, which triggers low-grade inflammation and results in insulin resistance. A chronic infusion of LPS over a 4-wk period in chow-fed mice leads to increased adiposity, increased macrophage infiltration in adipose tissues, hepatic inflammation, and hepatic insulin resistance[42]. Clinical trials have shown that SG decreases LPS levels[43], consistent with the outcome reported in our study. What is more, we found that LPS levels in the SG-OF group were lower than those in the SG group at 12 and 24 wk after surgery. A similar effect of oligofructose has also been observed in obese mice without surgery[15]. Therefore, we consider that the protective effect of oligofructose might partially be ascribed to the reduced LPS level.

The gut microbiota has been verified as an environmental factor affecting obesity and diabetes, and can modulate the metabolism of host glucose and lipid[44]. A recent study has revealed a significantly decreased *Firmicutes* abundance in T2DM patients when compared with nondiabetic individuals[45]. Meanwhile, a remarkably higher relative abundance of *Firmicutes* and a lower relative abundance of *Bacteroidetes* in rats receiving SG were observed. These results are consistent with the report from Ryan *et al*[46]. However, no difference in the relative abundances of *Firmicutes* and *Bacteroidetes* between the SG group and SG-OF group were observed, indicating that the benefit of oligofructose is not associated with the relative abundance of *Firmicutes* and *Bacteroidetes*. Notably, the rats from the SG-OF group had remarkably higher relative abundances of *Bifidobacterium*, *Lactobacillus*, and *Akkermansia muciniphila*. And the increased relative abundance of these bacteria has been observed in oligofructose-fed diabetic rodents by other research groups[19,47]. The advantage of oligofructose in the control of diabetes may be associated with an improvement in inflammation. Therefore, the effect of oligofructose in protecting the recurrence of diabetes after SG may be partially mediated by the increased abundance of *Bifidobacterium*, *Lactobacillus* and *Akkermansia muciniphila*, accompanied by the decrease in the abundance of *Bacteroides*, thus reducing the concentration of LPS.

There are still some limitations in this study. First, although oligofructose partially prevented the HFD-induced glucose and lipid metabolism damage after SG in rats, further clinical studies are highly desired to validate these results in humans. Second, the effects of oligofructose on calorie intake, insulin levels, GLP-1 levels, TBA levels, LPS levels, and the gut microbiota after SG were analyzed in this study; however, the intrinsic mechanism underlying the protective function of oligofructose should be further explored. We plan to further study the internal mechanism of these changes, especially focusing on the changes of the gut microbiota. At the same time, we are applying for relevant clinical trials to further observe the effects and side effects of probiotics, and we hope that oligofructose will become a novel target to reduce or delay the recurrence of diabetes after bariatric surgery.

CONCLUSION

Oligofructose can partially prevent the HFD-induced glucose and lipid metabolism damage in rats after SG, and the effects of oligofructose on calorie intake, insulin levels, GLP-1 levels, LPS levels, and the gut microbiota may contribute to this protective function.

ARTICLE HIGHLIGHTS

Research background

Type 2 diabetes mellitus is one of the important complications of obesity. Bariatric surgery can treat obesity and diabetes effectively, but the recurrence of diabetes after surgery is still one of the problems to be solved. However, whether oligofructose has an effect on metabolism after bariatric surgery remains to be further studied.

Research motivation

Prebiotics promote the weight loss and improve the metabolism of glucose and lipid. The mechanism of these benefits may be due to reduced energy intake, regulation of gut microbiota, improvement of low-grade inflammation, and increase of intestinal hormones, such as glucagon like peptide-1 (GLP-1) and peptide YY, which prompted us to explore whether prebiotics can reduce or delay the recurrence of diabetes after

surgery.

Research objectives

The present study aimed to find the effect and mechanism of oligofructose on diabetic remission in diabetic rats after sleeve gastrectomy (SG).

Research methods

SG and SHAM operation were performed on diabetes rats induced with a high-fat diet (HFD), nicotinamide, and low-dose streptozotocin. Then the rats in SHAM and SG groups were continuously provided with the HFD, and the rats in the SG-oligofructose group were provided with a specific HFD containing 10% oligofructose. Body weight, calorie intake, oral glucose tolerance test, homeostasis model assessment of insulin resistance, lipid profile, serum insulin, GLP-1, total bile acids, lipopolysaccharide (LPS), and colonic microbiota levels were determined and compared at the designated time points. All statistical analyses was performed using Solutionsstatistical Package for the Social Sciences version 19.0 (IBM, United States), and the statistically significant difference was considered at $P < 0.05$.

Research results

Oligofructose treatment reduced body weight, energy intake, and LPS levels, increased GLP-1 levels, and improved insulin resistant and lipid profiles. Oligofructose also altered the gut microbiota in the SG-oligofructose group.

Research conclusions

Oligofructose partially prevents HFD-induced glucose and lipid metabolism damage after SG in rats, and the effects of oligofructose on calorie intake, insulin, GLP-1, LPS, and gut microbiota may contribute to protective function for damaged metabolism.

Research perspectives

The results of this study provide evidence that oligofructose could be a promising agent for the treatment in diabetic rats after SG. Further studies that assess the effect of oligofructose on the mechanism of preventing HFD-induced glucose and lipid metabolism damage after SG may substantiate our findings and pave the path for clinical translation of the therapeutic effects of oligofructose.

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ORIGINAL ARTICLE

Basic Study

Elevated retinol binding protein 4 levels are associated with atherosclerosis in diabetic rats *via* JAK2/STAT3 signaling pathway

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Author contributions: All authors contributed significantly to the study; Zhou W designed the research, analyzed the data, and wrote the manuscript; Ye SD and Wang W reviewed and edited the manuscript; Zhou W and Wang W are the guarantors of this work.

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Institutional review board statement: The study was approved by The Ethics Committee of The First Affiliated Hospital of USTC. All procedures were performed in accordance with the ethical standards of the Declaration of Helsinki and its subsequent amendments or comparable ethical standards.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the

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Abstract

BACKGROUND

Atherosclerosis is a major cause of mortality worldwide and is driven by multiple risk factors, including diabetes, which results in an increased atherosclerotic burden, but the precise mechanisms for the occurrence and development of diabetic atherosclerosis have not been fully elucidated.

AIM

To summarize the potential role of retinol binding protein 4 (RBP4) in the pathogenesis of diabetic atherosclerosis, particularly in relation to the RBP4-Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway.

METHODS

Male Wistar rats were randomly divided into three groups, including a control group (NC group), diabetic rat group (DM group), and diabetic atherosclerotic rat group (DA group). The contents of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG), low-density lipoprotein cholesterol (LDL-c), fasting insulin (FINS), fasting plasma glucose, and hemoglobin A1c (HbA1c) were measured. Moreover, the adipose and serum levels of RBP4, along with the expression levels of JAK2, phosphorylated JAK2 (p-JAK2), STAT3, phosphorylated STAT3 (p-STAT3), B-cell lymphoma-2 (Bcl-2), and Cyclin D1 in

Institutional Animal Care and Use Committee of The First Affiliated Hospital of USTC (approval No. 20200049).

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aortic tissues were also measured. Besides, homeostasis model assessment of insulin resistance (HOMA-IR) and atherogenic indexes (AI) were calculated.

RESULTS

Compared with the NC and DM groups, the levels LDL-c, TG, TC, FINS, HOMA-IR, RBP4, and AI were upregulated, whereas that of HDL-c was downregulated in the DA group ($P < 0.05$); the mRNA levels of *JAK2*, *STAT3*, Cyclin D1, and *Bcl-2* in the DA group were significantly increased compared with the NC group and the DM group; P-JAK2, p-JAK2/JAK2 ratio, p-STAT3, p-STAT3/STAT3 ratio, Cyclin D1, and Bcl-2 at protein levels were significantly upregulated in the DA group compared with the NC group and DM group. In addition, as shown by Pearson analysis, serum RBP4 had a positive correlation with TG, TC, LDL-c, FINS, HbA1C, p-JAK2, p-STAT3, Bcl-2, Cyclin D1, AI, and HOMA-IR but a negative correlation with HDL-c. In addition, multivariable logistic regression analysis showed that serum RBP4, p-JAK2, p-STAT3, and LDL-c were predictors of the presence of diabetic atherosclerosis.

CONCLUSION

RBP4 could be involved in the initiation or progression of diabetic atherosclerosis by regulating the JAK2/STAT3 signaling pathway.

Key Words: Diabetes mellitus; Petinol binding protein 4; Atherosclerosis; JAK2/STAT3 signaling pathway; Cyclin D1

Core Tip: Atherosclerosis is a major cause of mortality worldwide and is driven by multiple risk factors including diabetes, which entails increased atherosclerotic burden, but the precise mechanisms for the occurrence and development of diabetic atherosclerosis are yet to be fully made clear. Retinol binding protein 4 is clinically associated with obesity, insulin resistance, type 2 diabetes, and cardiovascular diseases. This study aimed to explore the expression regulation and mechanism of retinol binding protein 4 that is involved in diabetic macrovascular disease in order to find therapeutic targets for diabetic macrovascular disease.

Citation: Zhou W, Ye SD, Wang W. Elevated retinol binding protein 4 levels are associated with atherosclerosis in diabetic rats *via* JAK2/STAT3 signaling pathway. *World J Diabetes* 2021; 12(4): 466-479
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INTRODUCTION

Atherosclerosis is a chronic disorder that involves inflammatory cell recruitment, endothelial dysfunction, local cytokine generation, and lipid accumulation in the vascular wall intima[1]. Retinol binding protein 4 (RBP4) is a novel adipokine released from hepatocytes and adipocytes and is considered as an emerging cardiometabolic risk factor that is correlated with obesity, insulin resistance (IR), impaired glucose tolerance, and type 2 diabetes (T2DM). This molecule was first reported by Yang *et al*[2], who, using a mouse model, found that the increased RBP4 expression in circulation resulted in IR by suppressing the activity of phosphatidylinositol 3-kinase (PI3K) in the skeletal muscle while upregulating the hepatic level of phosphoenol-pyruvate carboxylase. Increasing experimental and clinical studies have shown that upregulated RBP4 are positively associated with the prevalence of cardiovascular diseases including strokes, coronary heart disease, and hyper-tension[3-5], which thus indicates that RBP4 has a pivotal function in the mediation of cardiovascular diseases. However, the exact role of RBP4 in the occurrence and development of atherosclerosis remains elusive.

Recent studies have shown that the increased proliferation and migration of

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vascular smooth muscle cells (VSMCs) are a key event in the progression of cardiovascular diseases[6]. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which can regulate various pathophysiological processes, has been implicated in mediating cell migration and proliferation in VSMCs. Binding of cytokines to their receptors induces their autoactivation through transphosphorylation. Once activated, JAK2 is then rapidly activated in VSMCs, and STAT3 is phosphorylated and translocated to the nucleus in a JAK2-dependent manner[7].

Therefore, the aim of the present study was to determine the expression and function of RBP4 in diabetic rats with atherosclerosis, and to confirm whether the role of RBP4 in the development of atherosclerosis is mediated *via* the JAK/STAT signaling pathway.

MATERIALS AND METHODS

Animals

The study was approved by the Ethics Committee of Anhui Provincial Hospital Medical Institution Animal Care and Research Advisory Committee (Hefei, China) and was carried out in compliance with the Animal Research: Reporting *in vivo* Experiments Guidelines (ARRIVE Guidelines). Altogether 70 2-mo-old male Wistar rats weighing 190-210 g were purchased from the Experimental Animal Center of Anhui Medical University and raised in clean plastic cages at a temperature of 20 ± 1 °C, humidity of 47% ± 9%, and photoperiod of 14 h (light)/10 h (dark) during the entire experiment. The animals were divided into a normal control (NC, n = 20) or observation (n = 50) group. The NC group was given a normal diet, whereas the remaining animals were given a high-glucose and high-fat diet composed of 78.3% carbohydrates, 10% lard, 6% sugar, 5% cholesterol, 0.2% propylthiouracil, and 0.5% sodium cholate. The rats were acclimatized to the experimental conditions, and then, 30 mg/kg streptozotocin (STZ; Sigma-Aldrich, United States) was used to induce diabetes in rats randomly selected from the observation group. To confirm T2DM, we used a glucometer (Roche, Diagnostics GmbH, Germany) to carry out glucose tolerance tests on the rats fasted for 12 h. As a result, the 50 rats were shown to have T2DM (fasting plasma glucose, FPG > 7.8 mmol/L). The observation group rats continued on the high-glucose-high-fat diet and were randomly divided into diabetic rats (DM group, n = 25) and diabetic rats with atherosclerosis (DA group, n = 25). The rats in the DA group were then intraperitoneally injected with recombinant RBP4 (ab109146, abcam, United Kingdom) at 3 μg/g every 12 h for 3 wk.

Collection of specimens

At the end of week 19, each rat was anaesthetized intraperitoneally with 2% sodium pentobarbital sodium at a dose of 30 mg/kg. Then, blood was sampled to detect the related serum component contents. Except for samples for hematoxylin and eosin (HE) staining and immunohistochemistry, samples for Western blot assays of JAK2, phosphorylated JAK2 (p-JAK2), STAT3, phosphorylated STAT3 (p-STAT3), Cyclin D1, and B-cell lymphoma-2 (Bcl-2) were obtained when the thoracic aorta was separated and used to extract mRNA and protein. In addition, visceral adipose tissue was extracted for the measurement of the *RBP4* mRNA level and the quantitative protein expression of RBP4. All methods were performed in accordance with the relevant guidelines and regulations.

Laboratory assays

The levels of low-density lipoprotein cholesterol (LDL-c), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), and triglycerides (TG) were detected with an automatic biochemical analyzer (Hitachi 7600-020, Japan). Additionally, the levels of fasting insulin (FINS) were tested with an insulin radioimmunoassay kit (Atom Hi-Tech, China). The level of serum RBP4 was evaluated by enzyme-linked immunosorbent assay (ELISA; BIOHJSW, United States). Whole hemoglobin A1c (HbA1C) was measured by affinity chromatography with an HbA1c radiometer (BIO-RAB-D10, United States). HOMA-IR (homeostasis model assessment insulin resistance) index was calculated according to the following equations: HOMA-IR = FINS × FPG/22.5, and atherogenic index (AI) was estimated by the formulas AI = TC - HDL-c/HDL-c.

Western blot analysis

BCA assays were conducted to determine the protein levels in cell lysates. After separation through 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), the separated proteins were transferred onto a PVDF (polyvinylidene fluoride) membrane. At 4 °C, the membrane was simultaneously exposed overnight to solution containing primary antibodies [1:300, anti-JAK2 (Bioss, China); 1:5000, anti-p-JAK2 (abcam, United States); 1:1000, anti-STAT3 (Bioss, China); 1:10000, anti-p-STAT3 (abcam, United States); 1:300, anti-Cyclin D1 (Bioss, China); 1:1000, anti- Bcl-2 (Bioss, China); 1:1000, anti-RBP4 (Bioss, China)]. After the membrane was washed with Tris-buffered saline (TBS)-0.1% Tween 20 solution four times every 5 min, it was incubated with the corresponding secondary antibodies conjugated to horseradish peroxidase (1:10000, Zs-BIO, China). Western blots were treated with an ECL (electro-chemiluminescence) detection kit (Thermo, United States) to induce the chemiluminescence signal (Thermo, United States), which was captured on X-ray film.

Real-time quantitative reverse transcription-polymerase chain reaction

TRIzol (Invitrogen, United States) was used to extract total RNA. Thereafter, the RevertAidTM First Strand cDNA Synthesis Kit (Thermo, United States) was used to prepare cDNA in accordance with specific protocols. Moreover, the Novostart SYBR qPCR SuperMix Plus (Novoprotein, China) was adopted for real-time quantitative polymerase chain reaction (PCR) by using the fluorescence quantitative LightCycler 96 Real-Time PCR System (Thermo, United States). The primer sequences are listed in Table 1. The relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay

The serum RBP4 expression was detected by ELISA in accordance with specific instructions. After sample collection and standard preparation, the prepared products were added to specific wells. Thereafter, the substrate solution, together with detection reagents A and B, was added, for incubation at 37 °C for 30 min. Then, the stop solution was added to terminate the reaction, and the absorbance (OD) value was detected at 450 nm using a microplate reader.

Immunohistochemistry

The fixed tissue was washed, cleared, dehydrated, paraffin embedded, and cut into sections. Each tissue section was subjected to deparaffinization and treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidases, followed by 30 min of heating at 120 °C in 10 mmol/L citrate buffer to retrieve the antigen. Subsequently, primary antibodies dissolved in the phosphatebuffered saline (PBS) were added to incubate the sections at 4 °C overnight. The sections were then washed with PBS thrice, followed by 20 min of incubation with secondary antibodies and visualization with diaminobenzidine. Hematoxylin was then used for the counterstaining of each section. Ten visual fields were randomly chosen to take photographs (OLYMPUS, CX43) under a microscope, and the proportion of positively stained area to one visual field area (expressed as %) was determined for semiquantitative analysis.

Statistical analysis

Variables are presented as the mean ± SD or median. Two groups were compared by *t*-tests, and multiple groups were analyzed by one-way analysis of variance (ANOVA). Pearson's correlation coefficient was used to assess the relationship between RBP4 and other markers. Multivariable logistic regression analysis was used to calculate the odds ratios and 95% confidence intervals for diabetic atherosclerosis; $P < 0.05$ was considered statistically significant. All statistical analyses were carried out with SPSS 23.0 statistical software (IBM, Armonk, NY, United States).

RESULTS

Changes in biochemical indexes of rats in each group

As depicted in Table 2, the levels of FPG, HbA1C, TG, LDL-c, FINS, RBP4, AI, and HOMA-IR increased, while the level of HDL-c decreased in the DM and DA groups compared to the NC group ($P < 0.05$). When compared with the DM group, the levels of LDL-c, TG, TC, FINS, HOMA-IR, RBP4, and AI were significantly increased; conversely, the level of HDL-c was increased.

Table 1 Primer sequences of target genes and internal reference genes

Gene	Amplicon size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
β-actin	150	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC
Cyclin D1	138	TCAAGTGTGACCCGGACTG	GACCAGCTTCTTCCTCCACTT
STAT3	115	GCAATACCATTGACCTGCCG	AACGTGAGCGACTCAAACTG
RBP4	131	GCGAGGAAACGATGACCACT	TGGGGTCACGAGAAAACACA
JAK2	179	ACAAGCAGGACGGGAAGGTC	AATTGGGCCGTGACAGTTGC
Bcl2	102	GAGTACCTGAACCGGCATCT	GAAATCAAACAGAGGTCGCA

Table 2 Comparison of indicators among each group

Index	Group NC (*n* = 20)	Group DM (*n* = 25)	Group DA (*n* = 25)	*F*	*P* value
Weight (kg)	491.84 ± 80.82	504.61 ± 63.16	526.07 ± 85.66	1.155	0.321
TG (mmol/L)	0.65 ± 0.10	1.36 ± 0.17[a]	1.82 ± 0.28[a,b]	191.339	< 0.001
LDL-c (mmol/L)	0.35 ± 0.12	0.49 ± 0.12[a]	0.57 ± 0.14[a,b]	17.562	< 0.001
HDL-c (mmol/L)	1.07 ± 0.19	0.98 ± 0.20	0.71 ± 0.11[a,b]	27.856	< 0.001
TC (mmol/L)	1.98 ± 0.39	2.23 ± 0.42	2.95 ± 0.50 [a,b]	30.234	< 0.001
FPG (mmol/L)	5.42 ± 0.82	13.37 ± 2.16[a]	14.04 ± 2.40[a]	125.463	< 0.001
FINS (mU/L)	9.84 ± 1.99	14.42 ± 2.12[a]	19.24 ± 3.17[a,b]	77.990	< 0.001
HbA1C (%)	5.10 ± 0.81	9.95 ± 2.02[a]	10.86 ± 1.65[a]	78.800	< 0.001
RBP4 (ng/mL)	15.37 ± 2.07	21.23 ± 2.70[a]	32.28 ± 4.68[a,b]	144.583	< 0.001
AI	0.58 ± 0.23	1.71 ± 0.71[a]	3.31 ± 0.76[a,b]	105.551	< 0.001
HOMA-IR	2.38 ± 0.62	8.49 ± 1.36[a]	12.24 ± 2.82[a,b]	149.994	< 0.001

[a]$P < 0.05$ *vs* control group.
[b]$P < 0.05$ *vs* diabetic rat group. NC: Control group; DM: Diabetic rat group; DA: Diabetic atherosclerotic rat group; TG: Triglycerides; LDL-c: Low-density lipoprotein cholesterol; HDL-c: High-density lipoprotein cholesterol; TC: Total cholesterol; FPG: Fasting plasma glucose; FINS: Fasting insulin; HbA1C: Hemoglobin A1c; RBP4: Retinol binding protein 4; AI: Atherogenic indexes; HOMA-IR: Homeostasis model assessment of insulin resistance.

HE staining in each group

As illustrated in Figure 1A-C, the vessels had no obvious intimal thickening or lumen stenosis in the NC group. However, the intima became thicker and the structure and arrangement of VSMCs were disordered in the DM group. The lumen became narrower and a large number of VSMCs migrated and proliferated in the DA group.

Expression of RBP4 in adipose tissue of rats in each group

As shown in Figure 2, the mRNA expression of *RBP4* in adipose tissue was higher in the DA group (1.85 ± 0.17) than in the NC group (1.0 ± 0.08) and the DM group (1.58 ± 0.10). As illustrated in Figure 3, the protein expression of RBP4 was low in the NC group but dramatically increased in the DM and DA groups, and the increase was more significant in the DA group.

Expression of JAK2, STAT3, Cyclin D1, and Bcl-2 in aortic tissues of rats in each group

mRNA expression: The data revealed that the mRNA expression of *JAK2* in aortic tissues was 0.60 ± 0.02, 0.81 ± 0.03, and 0.99 ± 0.11 in the NC, DM, and DA groups, respectively. The mRNA expression of *STAT3* in aortic tissues was higher in the DA group (1.0 ± 0.08) than in the NC group (0.37 ± 0.06) and the DM group (0.92 ± 0.08). In addition, the mRNA expression of Cyclin D1 (1.0 ± 0.15) and Bcl-2 (1.67 ± 0.11) in the DA group was significantly increased compared with the NC group (Cyclin D1: 0.5 ± 0.08; Bcl-2: 0.85 ± 0.03) and the DM group (Cyclin D1: 0.82 ± 0.09; Bcl-2: 1.25 ± 0.05) (Figure 4).

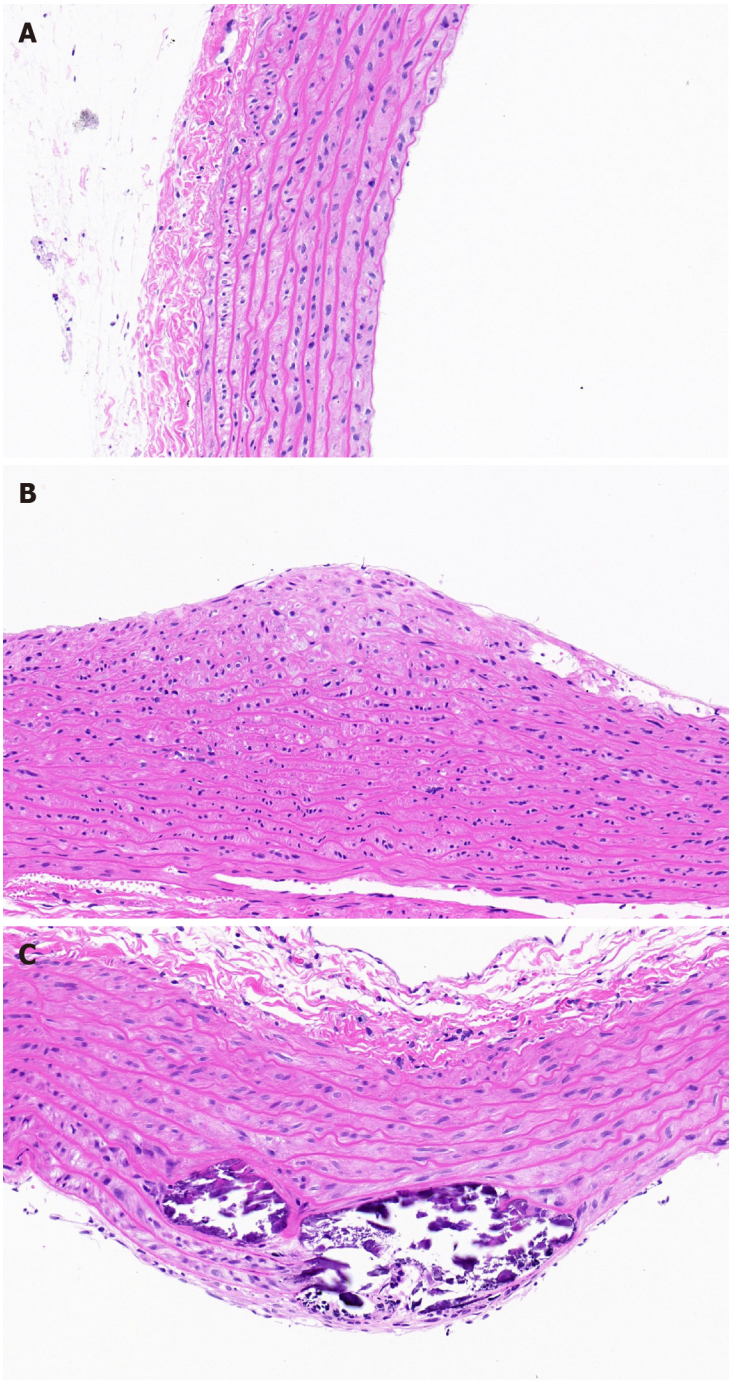

Figure 1 Thoracic aorta (hematoxylin and eosin staining). A: The vessel had no obvious intimal thickening and lumen stenosis in the control group; B: The intima became thicker and the structure and arrangement of vascular smooth muscle cells were disordered in the diabetic rat group; C: The lumen became narrower and a large number of vascular smooth muscle cells migrated and proliferated in the diabetic atherosclerotic rat group.

Protein expression by Western blot: P-JAK2, the p-JAK2/JAK2 ratio, p-STAT3, and the p-STAT3/STAT3 ratio were significantly increased in the DA group compared with the NC group and the DM group, while there was no significant difference in JAK2 and STAT3 expression among the groups (Figure 5A and B). The protein expression levels of Cyclin D1 and Bcl-2 in the DA group were significantly increased compared with the NC group and the DM group (Figure 5C).

Protein expression by immunohistochemistry: The positive protein expression of Cyclin D1 distributed in the nucleus (Figure 6A-C) and that of Bcl-2 distributed in the cytoplasm (Figure 6D-E), which were stained brown-yellow, were highly expressed in the aortic tissues of the rats in the DA group but were dramatically decreased in the DM and NC groups. The corresponding statistics are recorded in Table 3.

Table 3 Immunohistochemical analysis of Cyclin D1 and B-cell lymphoma-2 protein expression in all groups

Index	Group NC (%)	Group DM (%)	Group DA (%)	*F*	*P* value
Cyclin D1	6.84 ± 1.03	10.24 ± 1.78[a]	14.71 ± 2.26[a,b]	15.017	0.005
Bcl-2	7.25 ± 0.96	10.44 ± 1.21[a]	13.28 ± 2.04[a,b]	12.510	0.007

[a]*P* < 0.05 *vs* control group.
[b]*P* < 0.05 *vs* diabetic rat group.
NC: Control group; DM: Diabetic rat group; DA: Diabetic atherosclerotic rat group; Bcl-2: B-cell lymphoma-2.

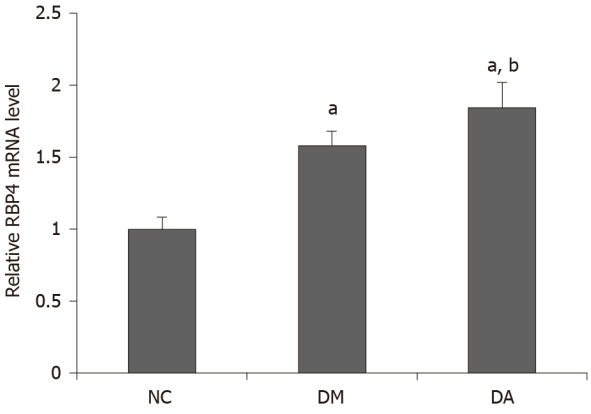

Figure 2 Comparisons of retinol binding protein 4 mRNA expression in each group. [a]*P* < 0.05 *vs* control group, [b]*P* < 0.05 *vs* diabetic rat group.
RBP4: Retinol binding protein 4; NC: Control group; DM: Diabetic rat group; DA: Diabetic atherosclerotic rat group.

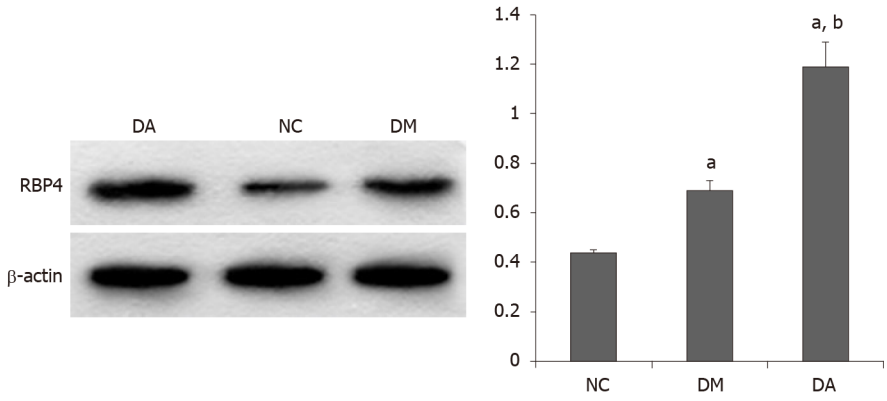

Figure 3 Comparisons of protein expression of retinol binding protein 4 in each group. [a]*P* < 0.05 *vs* control group, [b]*P* < 0.05 *vs* diabetic rat group.
RBP4: Retinol binding protein 4; NC: Control group; DM: Diabetic rat group; DA: Diabetic atherosclerotic rat group.

Correlation analysis and independent predictors of presence of atherosclerosis

As shown in Table 4, the results of correlation analysis revealed that serum RBP4 were positively correlated with TG, TC, LDL-c, FINS, HbA1C, p-JAK2, p-STAT3, Bcl-2, Cyclin D1, AI, and HOMA-IR but negatively correlated with HDL-c. Multiple logistic regression analysis was also used to determine the independent associations with the presence of diabetic atherosclerosis. The occurrence of atherosclerosis was set as a dependent variable, and the factors in the DM and DA groups were selected as independent variables. The results revealed that serum RBP4, LDL-c, p-JAK2, and p-STAT3 were risk factors for atherosclerosis (Table 5).

DISCUSSION

Atherosclerosis is a major cause of mortality worldwide and is driven by multiple risk

Table 4 Correlation between serum retinol binding protein 4 and the other indicators in type 2 diabetes groups (*n* = 50)

Indicator	*r*	*P* value
Weight (kg)	0.121	0.401
TG (mmol/L)	0.622	< 0.001
LDL-c (mmol/L)	0.395	0.005
HDL-c (mmol/L)	-0.566	< 0.001
TC (mmol/L)	0.586	< 0.001
AI	0.716	< 0.001
FPG (mmol/L)	0.111	0.444
FINS (mU/L)	0.556	< 0.001
HOMA-IR	0.541	< 0.001
HbA1C (%)	0.284	0.045
JAK2	0.239	0.094
p-JAK2	0.433	0.002
STAT3	-0.040	0.781
p-STAT3	0.539	< 0.001
Cyclin D1	0.476	< 0.001
Bcl-2	0.325	0.021

TG: Triglycerides; LDL-c: Low-density lipoprotein cholesterol; HDL-c: High-density lipoprotein cholesterol; TC: Total cholesterol; FPG: Fasting plasma glucose; FINS: Fasting insulin; HbA1C: Hemoglobin A1c; RBP4: Retinol binding protein 4; HOMA-IR: Homeostasis model assessment of insulin resistance; JAK2: Janus kinase 2; p-JAK2: Phosphorylated Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; p-STAT3: Phosphorylated signal transducer and activator of transcription-3; Bcl-2: B-cell lymphoma-2.

Table 5 Multivariable logistic regression in type 2 diabetes groups (*n* = 50)

Variable	*β* value	SE	χ^2	OR	95%CI	*P* value
RBP4	0.951	0.338	7.933	2.589	1.335-5.019	0.005
LDL-c	5.211	2.421	4.631	18.253	1.592-39.597	0.031
p-JAK2	2.040	0.866	5.554	7.693	1.410-41.979	0.018
p-STAT3	2.734	1.187	5.305	15.402	1.503-57.794	0.021
Constant	-21.007	7.916	7.041	0.000		0.008

CI: Confidence interval; OR: Odds ratio; RBP4: Retinol binding protein 4; LDL-c: Low-density lipoprotein cholesterol; p-JAK2: Phosphorylated Janus kinase 2; p-STAT3: Phosphorylated signal transducer and activator of transcription-3.

factors, including diabetes, which results in an increased atherosclerotic burden, but the precise mechanisms for the occurrence and development of diabetic atherosclerosis have not been fully elucidated. Dyslipidemia and inflammation have important synergistic effects in deteriorating vascular walls when diabetic atherosclerosis develops[8]. In addition, VSMCs play a critical role in various processes, including abnormal cell migration and proliferation, and synthesis of the extracellular matrix, which contribute to the progression of diabetic atherosclerosis[9]. JAK2/STAT3 is an important signal transduction pathway that mediates VSMC proliferation[10,11]. STAT3 can be activated when the signal transduction protein JAK2 is stimulated by cytokines to phosphorylate the STAT3 tyrosine 705 residue. Consequently, activated STAT3 forms dimers, translocates to the nucleus, and binds to downstream targets such as Bcl-2, Cyclin D1, c-Myc, and survivin to promote the proliferation and differentiation of VSMCs[12,13]. As previously reported, the JAK/STAT signal transduction pathway participates in the regulation of the inflammatory response of the arterial adventitia in diabetic patients. Banes *et al*[14] observed an increase in JAK2 and STAT3 tyrosine

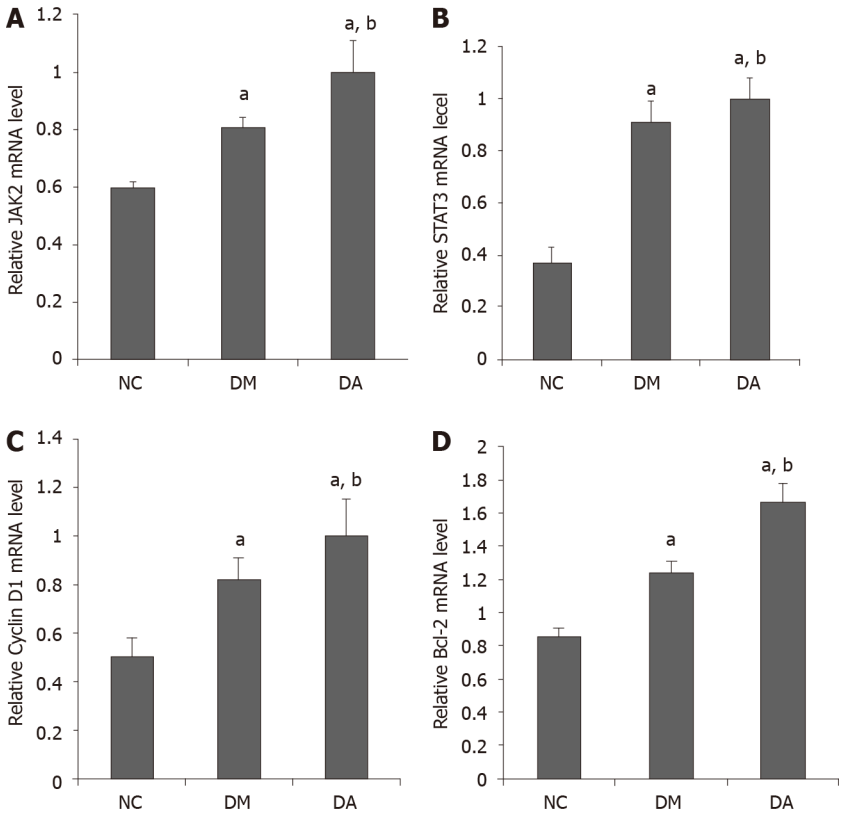

Figure 4 Comparisons of mRNA expression in each group. A: Janus kinase 2; B: Signal transducer and activator of transcription 3; C: Cylin D1; D: B-cell lymphoma-2. [a]*P* < 0.05 *vs* control group, [b]*P* < 0.05 *vs* diabetic rat group. NC: Control group; DM: Diabetic rat group; DA: Diabetic atherosclerotic rat group; JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; Bcl-2: B-cell lymphoma-2.

phosphorylation levels in STZ-induced rat thoracic aortae. These researchers also found that the treatment of diabetic rats with ketanserin prevented the increase in p-STAT3 and p-JAK2 levels in the aorta [14]. Recent studies have indicated that mice with endothelial STAT3 knockdown display decreased atherosclerotic lesions, lessened formation of fatty streaks, and intensified staining for activated STAT3 within inflammatory areas in atherosclerotic lesions relative to regions in their wild-type counterparts[15]. In the present study, both the mRNA and phosphorylated protein expression of JAK2 and STAT3 was significantly upregulated in the DA group compared with the NC group and the DM group, which was in accordance with previous studies.

RBP4 is clinically associated with obesity, IR, T2DM, and cardiovascular diseases. Furthermore, patients with diabetes complicated with macroangiopathy have enhanced serum RBP4 levels compared to diabetic patients with mild or no macroangiopathy[16-18], suggesting that RBP4 is involved in the pathogenesis of diabetes with macrovascular complications. As the causative factor and marker of vascular injury, RBP4 is related to IR[19]. Mohapatra *et al*[20] reported that concentrations of RBP4 in T2DM complicated with cardiovascular diseases were significantly increased and associated with glycolipid imbalances. RBP4 can also promote atherogenesis by inducing macrophage-derived foam cell formation[21]. As reported by Zabetian-Targhi *et al*[22], holo-RBP4 and apo-RBP4 result in the production of specific risk markers for inflammation and cardiovascular diseases, such as interleukin-6 and tumor necrosis factor-alpha in macrophages as well as vascular cell adhesion molecule-1 and intercellular cell adhesion molecule-1 in endothelial cells. Furthermore, RBP4 may directly induce cardiovascular diseases through inflammatory pathways. In our previous research, we found that RBP4 may contribute to the development of diabetic atherosclerosis through some mechanisms, such as IR, inflammatory reactions, and glycolipid metabolic disorder[23]. There are few studies on the potential role of RBP4 in the development of cardiovascular diseases, except for inflammation, IR, and glycolipid metabolism disorder. Gao *et al*[24] showed that RBP4 can promote the proliferation of VSMCs through the PI3K/AKT signaling pathway, which further leads to the occurrence of atherosclerosis. Moreover, Li *et al*[25] found that insulin induced the proliferation of VSMCs through the JAK2/STAT3 pathway and that RBP4

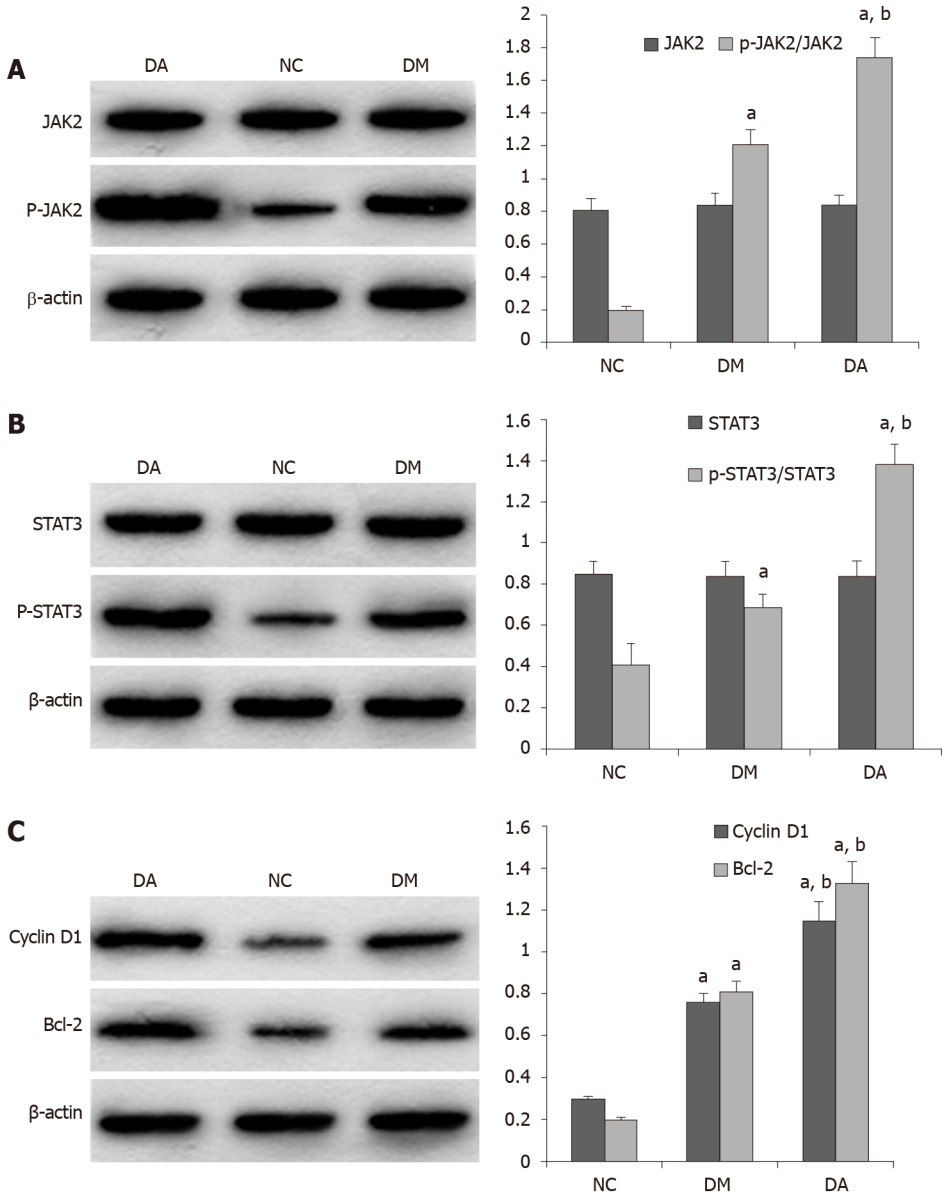

Figure 5 Comparisons of protein expression in each group. A: Janus kinase 2 and phosphorylated Janus kinase 2; B: Signal transducer and activator of transcription 3 and phosphorylated signal transducer and activator of transcription 3; C: Cylin D1 and B-cell lymphoma-2. [a]*P* < 0.05 *vs* control group, [b]*P* < 0.05 *vs* diabetic rat group. NC: Control group; DM: Diabetic rat group; DA: Diabetic atherosclerotic rat group; JAK2: Janus kinase 2; P-JAK2: Phosphorylated Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; P-STAT3: Phosphorylated signal transducer and activator of transcription 3; Bcl-2: B-cell lymphoma-2.

significantly promoted the hyperinsulinism-induced proliferation of VSMCs. These two studies are both consistent with the hypothesis that RBP4 may activate proliferation and migration of VSMCs through the JAK/STAT pathway. We therefore aimed to verify whether the JAK2/STAT3 signal is responsible for RBP4-induced proliferation and migration in VSMCs. In the present study, signs of atherosclerosis were present in the rats after 3 wk of intraperitoneal recombinant RBP4 injection. Statistical analysis also showed that RBP4 was positively correlated with p-JAK2, p-STAT3, Bcl-2, Cyclin D2, and AI and that RBP4 was one of the predictors of the presence of diabetic atherosclerosis, which implies that RBP4 can increase the degrees of phosphorylation of JAK2 and STAT3 and participate in the formation of atherosclerosis through the JAK2/STAT3 pathway. Previous studies have shown that elevated RBP4 may lead to IR and dyslipidemia through the JAK/STAT signaling pathway. It has been demonstrated that treatment of cultured adipocytes with RBP4 triggers the phosphorylation of retinoic acid gene 6 (STRA6), activation of JAK2 and STAT, and upregulation of suppressor of cytokine signaling 3 (SOCS3), leading to the suppression of insulin responses. Similarly, RBP4 injection in mice led to the activation of STRA6, resulting in the phosphorylation of JAK2 and STAT and the subsequent upregulation of SOCS3 expression in muscle and adipose tissue[26]. One study showed

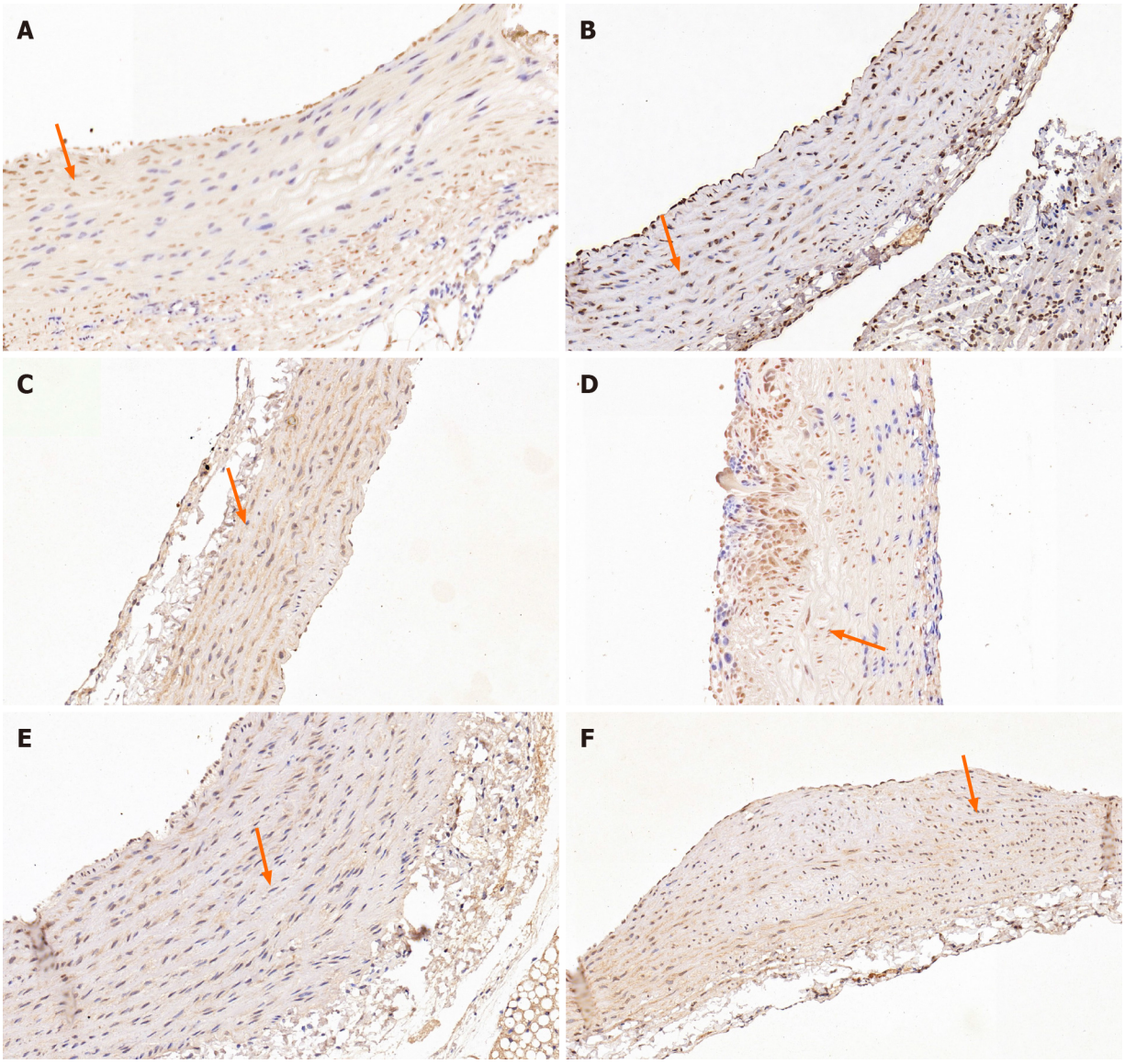

Figure 6 Protein expression by immunohistochemistry in each group. A: Cyclin D1 expression in control group; B: Cyclin D1 expression in diabetic rat group; C: Cyclin D1 expression in diabetic atherosclerotic rat group; D: B-cell lymphoma-2 (Bcl-2) expression in control group; F: Bcl-2 expression in diabetic rat group; G: Bcl-2 expression in diabetic atherosclerotic rat group.

that the RBP4/retinol complex stimulates the expression of SOCS3 through JAK/STAT signaling, which has been implicated in IR. These observations further revealed that activation of a JAK/STAT cascade by RBP-retinol leads to the upregulation of the expression of STAT target genes, which can inhibit insulin signaling or control lipid homeostasis[27]. Zargha *et al*[28] found that RBP4 can affect lipid metabolism through the JAK/STAT3 pathway mediated by STRA6. Leptin can affect vascular endothelial cell function and VSMC proliferation through the JAK/STAT pathway, which indicates that JAK2/STAT3 is an adipocyte-derived signaling pathway[29]. There are few studies investigating whether RBP4 can promote VSMC proliferation by using JAK/STAT signaling. We investigated the association between diabetic vascular complications and the RBP4-JAK2/STAT3 signaling pathway in STZ-induced diabetic rats and found that the expression of JAK2, STAT3, Bcl-2, and Cyclin D1 was increased after intraperitoneal injection of recombinant RBP4. Therefore, we speculate that RBP4 may be the upstream signal of the VSMC proliferation pathway by triggering the phosphorylation of JAK2 and STAT3, which leads to the upregulation of the expression of Bcl-2 and Cyclin D1 and accelerates the occurrence of diabetic atherosclerosis. It is important to explore the regulation and mechanism of RBP4 expression in diabetic macrovascular disease to find therapeutic targets for diabetic macrovascular disease.

CONCLUSION

In summary, increased secretion of RBP4 stimulates the expression of JAK2, STAT3, Bcl-2, and Cyclin D1 in diabetic rats and promotes the development of atherosclerosis. RBP4 may contribute to the development of diabetes complicated with cardiovascular disease, particularly through the RBP4-JAK2/STAT3 signaling pathway.

ARTICLE HIGHLIGHTS

Research background

With the increasing incidence of diabetes, the incidence of diabetic macroangiopathy continues to rise, which entails and increases atherosclerotic burden. Retinol binding protein 4 (RBP4) is clinically associated with obesity, insulin resistance, type 2 diabetes, and cardiovascular diseases. However, the precise role of RBP4 in the initiation and progression of atherosclerosis remains elusive.

Research motivation

We tried to provide new insight into the mechanism of diabetic atherosclerosis.

Research objectives

This study aimed to explore the expression regulation and mechanism of RBP4 in the diabetic rats with atherosclerosis, and to examine whether the role of RBP4 in the progression of atherosclerosis is mediated *via* the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway.

Research methods

Male Wistar rats were randomly divided into a control group (NC group), diabetic rats (DM group), and diabetic atherosclerosis rats (DA group). At the end of week 19, serum RBP4, fasting insulin (FINS), fasting plasma glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG), low-density lipoprotein cholesterol (LDL-c), and hemoglobin A1c were measured. Except for hematoxylin and eosin staining and immunohistochemistry, the thoracic aorta was separated and extracted for mRNA and Western blot assay of JAK2, phosphorylated-JAK2 (p-JAK2), STAT3, phosphorylated-STAT3 (p-STAT3), Cyclin D1, and B-cell lymphoma-2 (Bcl-2). In addition, visceral adipose tissue was extracted for the measurement of *RBP4* mRNA and the quantitative protein expression of RBP4. Homeostasis model assessment of insulin resistance (HOMA-IR) and atherogenic index (AI) were calculated.

Research results

Compared with the NC group and DM group, the levels of LDL-c, TG, TC, FINS, HOMA-IR, RBP4, and AI increased, while the level of HDL-c decreased in the DA group. The mRNA expression of *JAK2*, *STAT3*, Cyclin D1, and *Bcl-2* in the DA group was significantly increased compared with the NC group and DM group. P-JAK2, p-JAK2/JAK2 ratio, p-STAT3, p-STAT3/STAT3 ratio, Cyclin D1, and Bcl-2 in the DA group were significantly increased at protein levels compared with the NC group and DM group. Pearson analysis showed that serum RBP4 was positively correlated with TG, TC, LDL-c, FINS, hemoglobin A1c, p-JAK2, p-STAT3, Bcl-2, CyclinD1, AI, and HOMA-IR but negatively correlated with HDL-c. In addition, multivariable logistic regression analysis showed that serum RBP4, p-JAK2, p-STAT3, and LDL-c were predictors of the presence of diabetic atherosclerosis.

Research conclusions

The current study demonstrated that RBP4 could be involved in the initiation or progression of diabetic atherosclerosis by regulating the JAK2/STAT3 signaling pathway.

Research perspectives

These results provide important insights into the mechanism of diabetic atherosclerosis and may help find therapeutic targets for diabetic macrovascular disease.

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ORIGINAL ARTICLE

Basic Study

Vascular endothelial growth factor B inhibits insulin secretion in MIN6 cells and reduces Ca^{2+} and cyclic adenosine monophosphate levels through PI3K/AKT pathway

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Author contributions: Li YN and Tian G conceived and designed the study; Jia JD, Luo X, and Li RR performed the experiments; Jiang WG and Zhao YC analyzed the data; Jia JD wrote the manuscript; Li YN revised the manuscript; all authors approved the final version of the article.

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Abstract

BACKGROUND

Type 2 diabetes (T2D) is characterized by insufficient insulin secretion caused by defective pancreatic β-cell function or insulin resistance, resulting in an increase in blood glucose. However, the mechanism involved in this lack of insulin secretion is unclear. The level of vascular endothelial growth factor B (VEGF-B) is significantly increased in T2D patients. The inactivation of VEGF-B could restore insulin sensitivity in db/db mice by reducing fatty acid accumulation. It is speculated that VEGF-B is related to pancreatic β-cell dysfunction and is an important factor affecting β-cell secretion of insulin. As an *in vitro* model of normal pancreatic β-cells, the MIN6 cell line can be used to analyze the mechanism of insulin secretion and related biological effects.

AIM

To study the role of VEGF-B in the insulin secretion signaling pathway in MIN6 cells and explore the effect of VEGF-B on blood glucose regulation.

METHODS

The MIN6 mouse pancreatic islet β-cell line was used as the model system. By administering exogenous VEGF-B protein or knocking down VEGF-B expression in MIN6 cells, we examined the effects of VEGF-B on insulin secretion, Ca^{2+} and cyclic adenosine monophosphate (cAMP) levels, and the insulin secretion signaling pathway.

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Conflict-of-interest statement: The authors declare that they have no conflicts of interest to disclose.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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Peer-review report's scientific quality classification

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Grade B (Very good): 0
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Grade D (Fair): 0
Grade E (Poor): 0

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RESULTS

Exogenous VEGF-B inhibited the secretion of insulin and simultaneously reduced the levels of Ca^{2+} and cAMP in MIN6 cells. Exogenous VEGF-B also reduced the expression of phospholipase C gamma 1 (PLCγ1), phosphatidylinositol 3-kinase (PI3K), serine/threonine kinase (AKT), and other proteins in the insulin secretion pathway. Upon knockdown of VEGF-B, MIN6 cells exhibited increased insulin secretion and Ca^{2+} and cAMP levels and upregulated expression of PLCγ1, PI3K, AKT, and other proteins.

CONCLUSION

VEGF-B can regulate insulin secretion by modulating the levels of Ca^{2+} and cAMP. VEGF-B involvement in insulin secretion is related to the expression of PLCγ1, PI3K, AKT, and other signaling proteins. These results provide theoretical support and an experimental basis for the study of VEGF-B in the pathogenesis of T2D.

Key Words: Type 2 diabetes; Insulin secretion; MIN6 cells; Vascular endothelial growth factor B; Blood glucose regulation

Core Tip: Type 2 diabetes has elicited worldwide public health concerns, and mechanism regulating insulin secretion is unclear. We found that vascular endothelial growth factor B (VEGF-B) prevents MIN6 cells from secreting insulin through the PI3K-AKT (phosphatidylinositol 3-kinase-serine/threonine kinase) pathway. We have provided mechanistic insights into the effect of VEGF-B on insulin secretion and suggest VEGF-B as a new target that affects the occurrence and development of type 2 diabetes.

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INTRODUCTION

Diabetes and diabetic complications are serious threats to human health. Due to the high fatality rate, disability rate, and medical costs, diabetes has become a common health problem faced by countries worldwide. The International Diabetes Federation predicts that by 2040, there will be 642 million adults with diabetes worldwide, of which more than 90% will comprise patients with type II diabetes, which is caused by insulin secretion defects or decreased insulin sensitivity[1]. However, the mechanism of insulin secretion is still not completely clear. Further exploration of the mechanism of insulin secretion has been an important topic in various disciplines and has attracted the attention of scholars worldwide.

In 2012, Hagberg *et al*[2] proposed for the first time that abnormal lipid deposits in peripheral tissues can impair insulin sensitivity, which in turn affects glucose uptake, and is one of the factors that triggers type 2 diabetes (T2D).

Researchers discovered that reducing vascular endothelial growth factor B (VEGF-B) levels in db/db mice could restore insulin sensitivity by reducing lipid accumulation in heart and skeletal muscle; blocking VEGF-B signaling in high-fat diet (HFD)-fed rats could also restore insulin sensitivity and increase glucose uptake in skeletal muscle and heart, thereby reducing blood glucose levels. Hagberg *et al*[2] proposed that VEGF-B is involved in the lipid uptake process, opening the possibility of new strategies for regulating pathological lipid accumulation in diabetes, obesity, and cardiovascular diseases[2]. In 2016, Mehlem *et al*[3] further confirmed that VEGF-B inactivation could reduce the accumulation of ectopic lipids, thereby improving insulin resistance. By contrast, high levels of VEGF-B can lead to the deterioration of insulin resistance and blood glucose control. In 2020, Ning *et al*[4] studied a mouse

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model of islet β cell-specific VEGF-B deficiency and found that the specific deletion of VEGF-B in islet β-cells could upregulate the expression of the insulin gene (*Ins2*) and increase insulin secretion.

Although the specific mechanism of VEGF-B involvement in insulin secretion and insulin resistance is not yet clear, studies have confirmed that VEGF-B affects insulin secretion by regulating fatty acid (FA) metabolism, and can improve obesity-related metabolic complications and restore insulin sensitivity[2,5]. VEGF-B belongs to the VEGF family. Compared with VEGF-A, VEGF-B does not regulate the growth of blood vessels but can regulate the uptake of endothelial FAs and promote the survival of cardiomyocytes, retinal neurons, and other cells, showing a proliferative effect on cells[3]. VEGF-B is mainly expressed in tissues with high metabolic activity, such as the myocardium, skeletal muscle, and brown fat. VEGF-B/VEGFR (VEGF receptor) signaling plays a certain role in inhibiting Ang II-induced cardiac hypertrophy[6,7], promoting lipid absorption in skeletal muscle, inhibiting FA re-esterification, and promoting triglyceride participation in the process of oxidation and decomposition[8]. At present, VEGF-B has been predicted to be a promising candidate to treat T2D, but there have been few studies on VEGF-B and insulin secretion. Some of the questions to be addressed include whether VEGF-B participates in glucose-induced insulin secretion or in regulating the signaling pathway of insulin secretion. Studies on these topics will provide the basis for understanding the role of VEGF-B in insulin secretion and diabetes.

This study therefore explored the role of VEGF-B in the insulin secretion signaling pathway and blood glucose regulation by interfering with the expression of VEGF-B in MIN6 cells. We observed changes in insulin secretion, Ca^{2+} and cyclic adenosine monophosphate (cAMP) levels, and the expression of proteins related to the insulin secretion signaling pathway. Studies have shown that exogenous VEGF-B protein inhibits the secretion of insulin in MIN6 cells, and insulin secretion is increased after knocking down VEGF-B. Moreover, the effect of VEGF-B on Ca^{2+} and cAMP is consistent with the changes in insulin secretion. We observed that VEGF-B affected the expression of VEGF receptor 1 (VEGFR1), neuropilin 1 (NRP1), phospholipase C gamma 1 (PLCγ1), phosphatidylinositol 3-kinase (PI3K), serine/threonine kinase (AKT), and other proteins. Thus, we determined that VEGF-B could participate in the insulin secretion by binding to VEGFR1 and NRP1 and consequently affecting the expression of proteins related to the insulin secretion signaling pathway.

MATERIALS AND METHODS

Cell culture

MIN6 mouse insulinoma cells (Wuhan Feien Biotechnology, China) were cultured in RPMI 1640 (HyClone, United States) supplemented with 10% fetal bovine serum (FBS; Gibco, Brazil), 1% penicillin, and 1% streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. VEGF-B protein (PeproTech, United States) treatment groups were divided into five concentrations: 0, 12.5, 25, 50, and 100 ng/mL. There were three small interfering RNA (siRNA) knockdown groups — Si1, Si2, and Si3 — according to the different Si-M-VEGF-B primer target sequences (Supplementary Table 1); a control group was also established.

Cell counting kit-8

Cell counting kit-8 (CCK-8) was used to detect the effects of exogenous VEGF-B and VEGF-B knockdown on the proliferation of MIN6 cells. MIN6 cells were seeded in a 96-well plate at a cell density of 2×10^5/mL. After culture in a cell incubator at 37 °C for 6 h, MIN6 cells were treated with exogenous VEGF-B protein or subjected to knockdown of VEGF-B protein. The original medium was discarded and the cells were washed with PBS buffer. Next, the medium was mixed with CCK-8 solution (Bimake, United States) at a ratio of 9:1 and 100 μL was added to each well. The plates were incubated for another 2 h before they were placed in a microplate reader (Bio Tek, United States) to detect the optical density at 450 nm.

siRNA transfection

After MIN6 cells were seeded in a 12-well plate and incubated for 12 h, they were transfected for 48 h with medium containing 500 nmol/L siRNA and jetPRIM reagent (Polyplus, France); the sequences of siRNAs used are listed in Supplementary Table 2. Then, the cells were used for different assays or lysed for RNA or protein isolation.

Quantitative reverse transcription-polymerase chain reaction

MIN6 cells were processed and total RNA was extracted. After reverse transcription with RNA-easy Isolation Reagent (Vazyme, China), 1000 ng of cDNA was obtained with the TB Green premix Ex Taq II kit (Takara, Japan) in a QuantStudio 3 PCR machine (Thermo Fisher, United States) for real-time polymerase chain reaction (PCR) amplification (Supplementary Tables 3 and 4).

Enzyme-linked immunoassay

Enzyme-linked immunoassay kit (ELISA) kits (mlbio, China) were used to detect insulin secretion and intracellular Ca^{2+} and cAMP contents in MIN6 cells. The supernatant was collected after cells were subjected to the indicated treatments. For insulin secretion, cells were centrifuged at 2000-3000 r/min for 20 min, and an Ins ELISA kit was used. For determining the intracellular Ca^{2+} and cAMP content, cells underwent repeated freeze/thaw cycles before they were centrifuged at 2000-3000 r/min for 20 min; the supernatant was collected and processed with Ca^{2+} and cAMP kits, respectively. The absorbance (OD value) was measured with a microplate reader at a wavelength of 450 nm, standard curves were established for each kit, and the insulin, Ca^{2+}, and cAMP levels of each group of samples were calculated from the respective standard curves. Statistical analysis was then performed on the resulting data.

Western blot analysis

The protein of MIN6 cells was extracted and the protein concentration was measured with a BCA kit (Takara, Japan). Specific antibodies against VEGFR1, NRP1, PI3K-p85, PI3K-p110γ, PLCγ1, phosphorylated (p)-PLCγ1, AKT, and p-AKT were used for the corresponding protein detection, and β-actin was used as the internal reference for normalization. Antibody information and concentration are listed in Supplementary Table 5. The antigens were visualized using an ECL plus detection system (Tanon-5200).

Flow cytometry

The effect of exogenous VEGF-B on MIN6 cell apoptosis was detected. After centrifugation at 2000 r/min, the cell density was adjusted to 5×10^5/mL. After treatment with the Apoptosis Detection Kit (KeyGEN BioTECH, China), apoptosis was detected on a flow cytometer (BD Canto II).

Statistical analysis

The data were statistically analyzed using SPSS 22 and are reported as the mean ± SE. One-way analysis of variance was used to analyze the differences between multiple groups, while the least-significant difference method was used to analyze normally distributed data. $P < 0.05$ indicated the statistical difference and $P < 0.001$ was considered statistically significant.

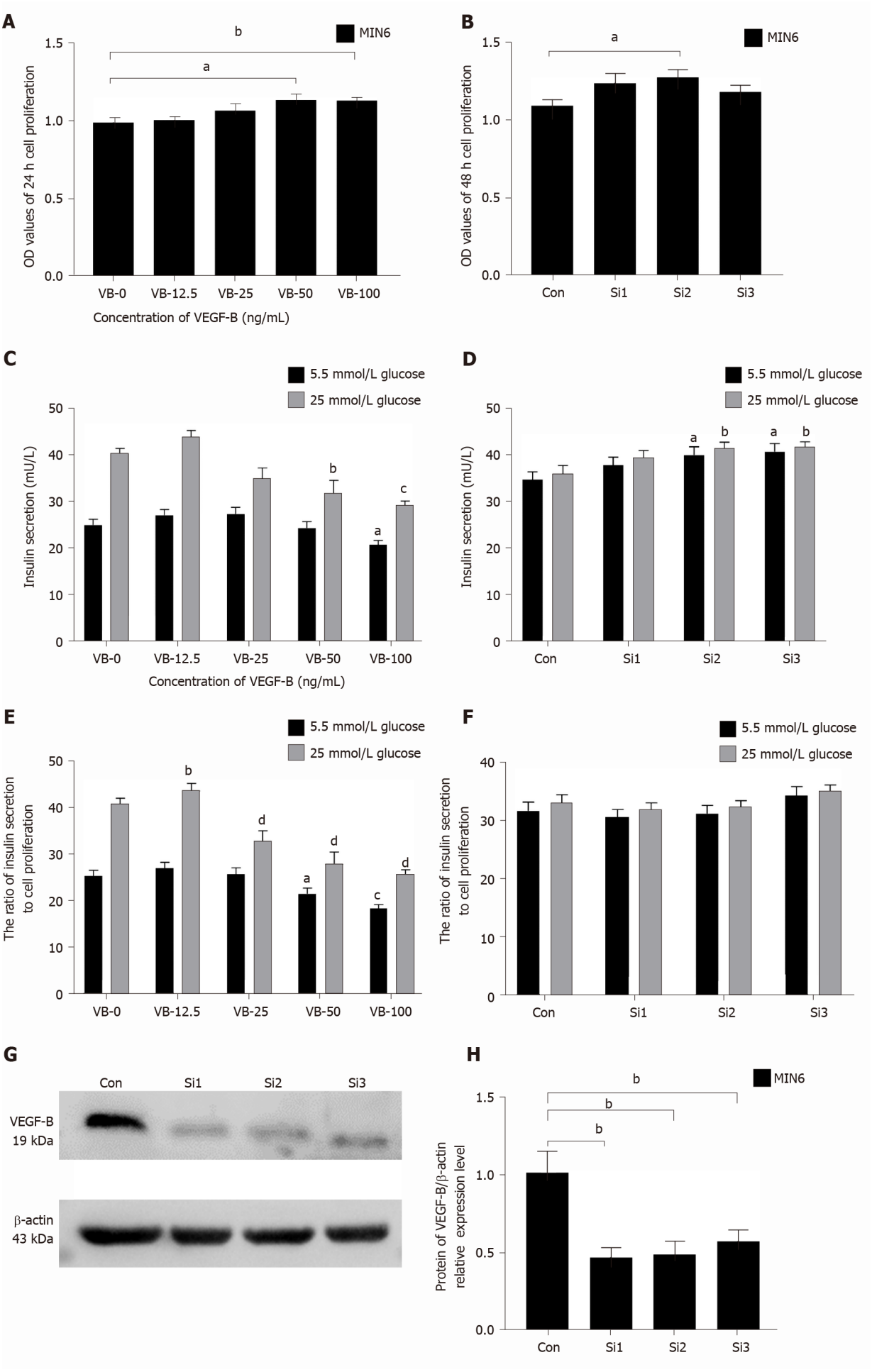
RESULTS

Effects of VEGF-B on insulin secretion in MIN6 cells

Among MIN6 cells stimulated with five concentrations of VEGF-B protein for 24 h, the number of MIN6 cells in the VB (VEGF-B)-50 and VB-100 group was significantly increased compared with that in the VB-0 group ($P < 0.05$ and $P < 0.01$, respectively) (Figure 1A). After VEGF-B expression in MIN6 cells was knocked down by siRNA, cell proliferation showed an increasing trend (Figure 1B).

Under the same VEGF-B protein treatment conditions, the insulin secretion of each group was measured. The results showed that as the concentration of VEGF-B protein increased, insulin secretion showed a decreasing trend. Among the groups, the VB-100 group showed a significantly lower level of insulin secretion in response to 5.5 mmol/L glucose than the control group ($P < 0.05$); in the medium containing 25 mmol/L glucose, the insulin secretion in the VB-50 and VB-100 groups was significantly reduced compared with the VB-0 group ($P < 0.01$ and $P < 0.001$, respectively) (Figure 1C). Upon siRNA-mediated knockdown of VEGF-B in MIN6 cells and subsequent culture in 5.5 mmol/L or 25 mmol/L glucose, insulin secretion in the Si2 and Si3 groups was significantly higher than that of the control (Con) group ($P < 0.05$ for both) (Figure 1D).

Insulin secretion per unit cell proliferation level in each group was analyzed. In a


A
MIN6
OD values of 24 h cell proliferation
VB-0
VB-12.5
VB-25
VB-50
VB-100
Concentration of VEGF-B (ng/mL)
B
MIN6
OD values of 48 h cell proliferation
Con
Si1
Si2
Si3
C
5.5 mmol/L glucose
25 mmol/L glucose
Insulin secretion (mU/L)
Concentration of VEGF-B (ng/mL)
D
5.5 mmol/L glucose
25 mmol/L glucose
Insulin secretion (mU/L)
E
5.5 mmol/L glucose
25 mmol/L glucose
The ratio of insulin secretion to cell proliferation
F
5.5 mmol/L glucose
25 mmol/L glucose
The ratio of insulin secretion to cell proliferation
G
Con
Si1
Si2
Si3
VEGF-B
19 kDa
β-actin
43 kDa
H
MIN6
Protein of VEGF-B/β-actin relative expression level

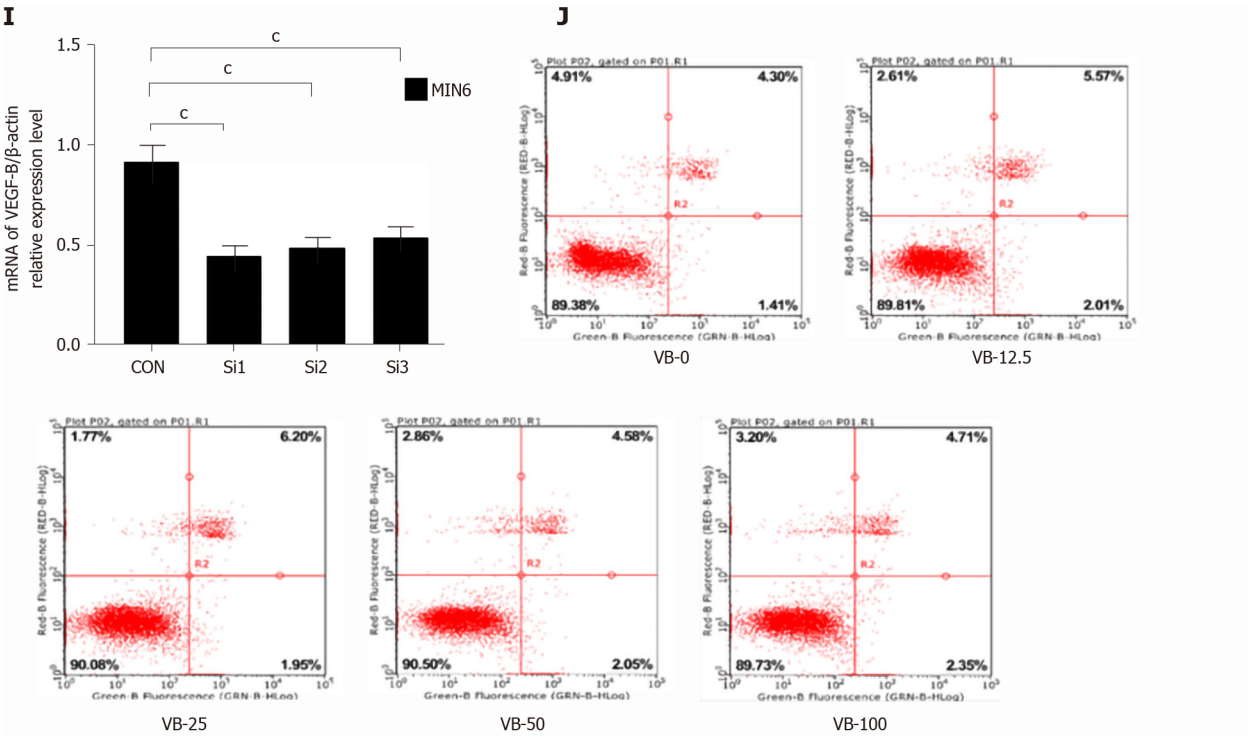


Figure 1 Decreased insulin secretion in MIN6 cells is related to vascular endothelial growth factor B function. A: Treatment with a gradient concentration of vascular endothelial growth factor B (VEGF-B, VB) protein promotes MIN6 cell proliferation. $^{a}P < 0.05$ *vs* VB-0, $^{b}P < 0.01$ *vs* VB-0; B: MIN6 cells with VEGF-B knockdown show an increasing trend in proliferation. $^{a}P < 0.05$ *vs* Control; C: Stimulation with a gradient concentration of VEGF-B protein inhibits insulin secretion by MIN6 cells. $^{a}P < 0.05$ *vs* VB-0 in the presence of 5.5 mmol/L glucose; $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* VB-0 in the presence of 25 mmol/L glucose; D: MIN6 cells with VEGF-B knockdown exhibit increased insulin secretion. $^{a}P < 0.05$ *vs* Control in the presence of 5.5 mmol/L glucose; $^{b}P < 0.05$ Control in the presence of 25 mmol/L glucose; E: The ratio of insulin secretion by MIN6 cells to their corresponding proliferation upon stimulation with a gradient concentration of VEGF-B protein. $^{a}P < 0.05$, $^{c}P < 0.001$ *vs* VB-0 in the presence of 5.5 mmol/L glucose; $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* Control in the presence of 25 mmol/L glucose; F: The ratio of insulin secretion in MIN6 cells to its corresponding cell proliferation after knocking down VEGF-B; G and H: Western blot analysis revealing relative VEGF-B protein expression in cells transfected with VEGF-B siRNA and corresponding statistical data. $^{b}P < 0.01$ *vs* Control; I: Reverse transcription-polymerase chain reaction revealing relative VEGF-B mRNA expression in cells transfected with VEGF-B siRNA and corresponding statistical data. $^{c}P < 0.001$ *vs* Control; J: Flow cytometry results indicating that VEGF-B does not affect the apoptosis of MIN6 cells. Student's *t*-test was performed. VEGF-B: Vascular endothelial growth factor B; VB: VEGF-B; Con: Control; Si: Small interfering RNA; MIN6: MIN6 cell; OD: Optical density.

medium containing 5.5 mmol/L glucose and after 24 h of treatment with different concentrations of VEGF-B, insulin secretion showed a decreasing trend, and insulin secretion in the VB-100 group was significantly reduced compared to that in the VB-0 group ($P < 0.05$). In the presence of 25 mmol/L glucose, VB-25 group had higher insulin secretion compared with the VB-0 group, whereas insulin secretion in the other groups was significantly reduced ($P < 0.001$) (Figure 1E).

After knocking down VEGF-B, there was no statistical difference in insulin secretion or cell proliferation ratio between the Con group and knockdown groups (Figure 1F).

Verification of siRNA knockdown of VEGF-B showed that the levels of mRNA and protein expression of VEGF-B in transfected MIN6 cells were significantly lower than those in the Con cells ($P < 0.01$ and $P < 0.001$, respectively) (Figure 1G-I).

The flow cytometry results showed that after 24 h of exogenous VEGF-B stimulation in MIN6 cells, the ratio of viable, early apoptotic, late apoptotic, and necrotic cells in the VEGF-B-treated groups (at different concentrations) was not statistically significant compared with the VB-0 group (Figure 1J).

Effects of exogenous VEGF-B on the expression of VEGFR1 and NRP1

Western blot analysis revealed that in MIN6 cells stimulated with exogenous VEGF-B for 24 h, when the VEGF-B protein concentration exceeded 25 ng/mL, the expression level of VEGFR1 protein was higher than that of the VB-0 group. The VB-50 and VB-100 groups showed statistical differences ($P < 0.05$). Except for the VB-25 treatment, all the VEGF-B-treated groups exhibited slightly higher protein expression levels of NRP1 than the VB-0 group (Figure 2A-C).

After undergoing siRNA-mediated knockdown of VEGF-B, MIN6 cells were cultured in 5.5 mmol/L and 25 mmol/L glucose for 24 h. Compared with those of the Con group, the expression levels of VEGFR1 and NRP1 were reduced in the VEGF-B-

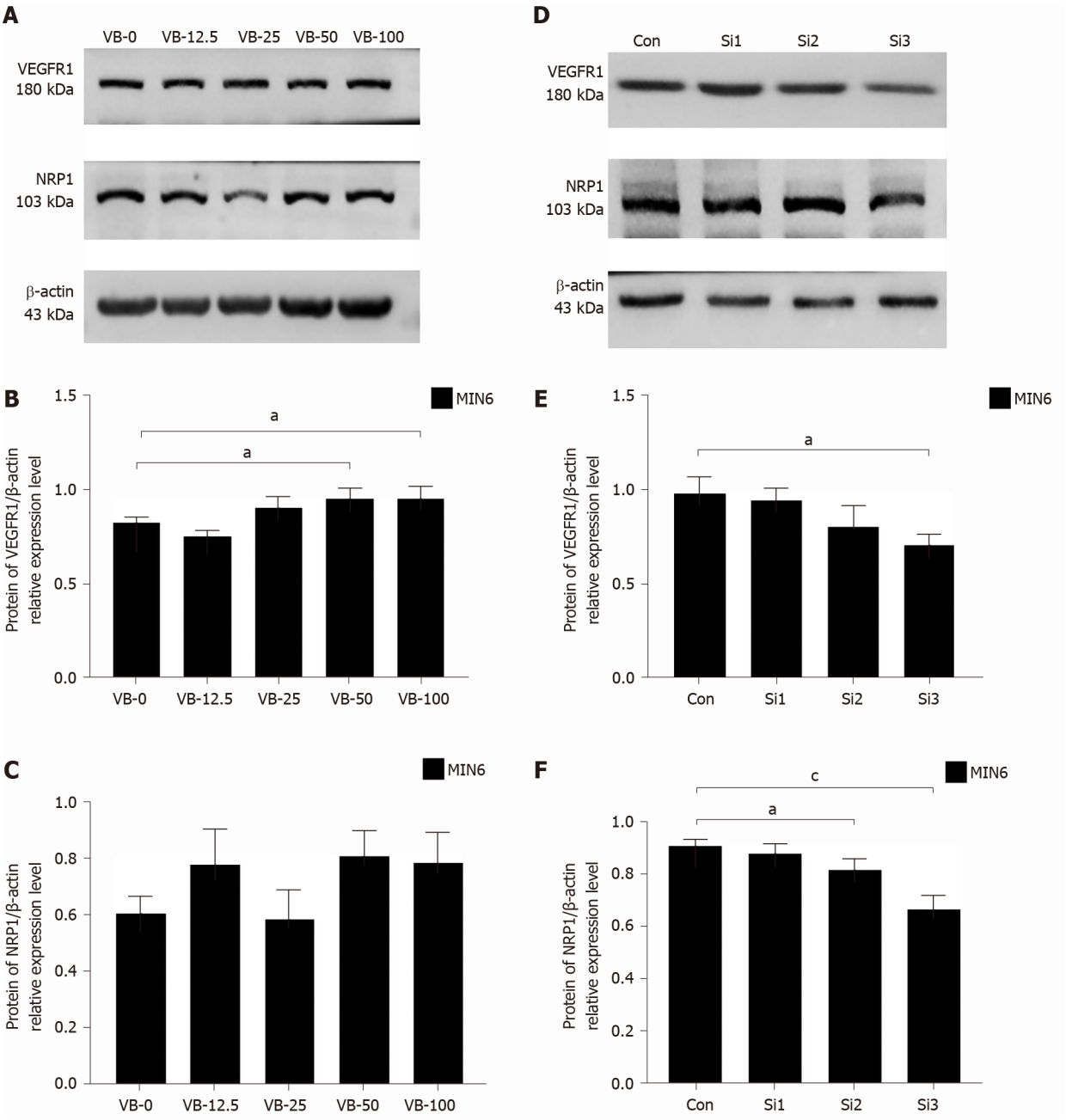


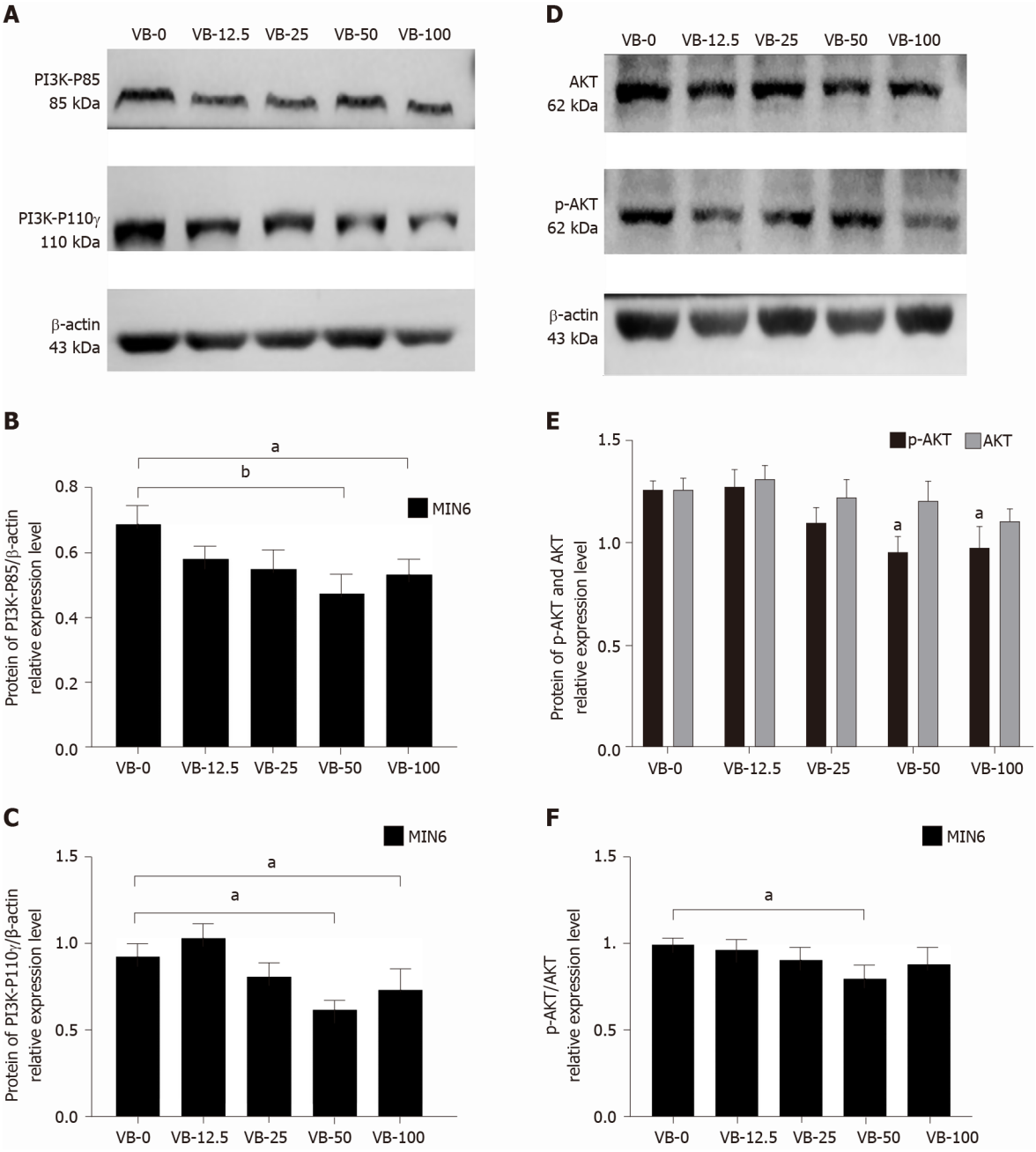
Figure 2 Exogenous vascular endothelial growth factor B stimulation increases the expression of vascular endothelial growth factor B receptor 1, while vascular endothelial growth factor B small interfering RNA transfection decreases the expression of vascular endothelial growth factor B receptor 1 and neuropilin 1. A-C: Western blot analysis revealing the relative protein expression of vascular endothelial growth factor B receptor 1 (VEGFR1) and neuropilin 1 in cells stimulated with exogenous VEGF-B and corresponding statistical data; D-F: Western blot analysis indicating the relative protein expression of VEGFR1 and neuropilin 1 in cells transfected with VEGF-B small interfering RNA and corresponding statistical data. Student's *t*-test was performed. $^{a}P < 0.05$ *vs* Control; $^{c}P < 0.001$ *vs* Control. NRP1: Neuropilin 1; VEGF-B: Vascular endothelial growth factor B; VB: VEGF-B; Con: Control; Si: Small interfering RNA; MIN6: MIN6 cell.

knockdown groups ($P < 0.05$ and $P < 0.001$, respectively; Figure 2D-F).

Effect of exogenous VEGF-B on the expression of PI3K-AKT pathway proteins

The expression levels of PI3K-p85 in MIN6 cells stimulated with gradient concentrations of VEGF-B protein for 24 h were lower than those of the VB-0 group, and those were significantly lowered in the VB-50 and VB-100 groups. ($P < 0.01$ and $P < 0.05$, respectively). Except for the VB-12.5 group, the protein expression levels of PI3K-p110γ in the remaining four groups were lower than that of the VB-0 group, and the decrease in the expression levels of PI3K-p110γ in the VB-50 and VB-100 groups was statistically significant ($P < 0.05$) (Figure 3A-C).

MIN6 cells were stimulated with VEGF-B for 24 h, and the expression levels of AKT


A
VB-0 VB-12.5 VB-25 VB-50 VB-100
PI3K-P85
85 kDa
PI3K-P110γ
110 kDa
β-actin
43 kDa
D
VB-0 VB-12.5 VB-25 VB-50 VB-100
AKT
62 kDa
p-AKT
62 kDa
β-actin
43 kDa
B
a
b
MIN6
Protein of PI3K-P85/β-actin relative expression level
0.0 0.2 0.4 0.6 0.8
VB-0 VB-12.5 VB-25 VB-50 VB-100
E
p-AKT AKT
Protein of p-AKT and AKT relative expression level
0.0 0.5 1.0 1.5
a a
VB-0 VB-12.5 VB-25 VB-50 VB-100
C
MIN6
a
a
Protein of PI3K-P110γ/β-actin relative expression level
0.0 0.5 1.0 1.5
VB-0 VB-12.5 VB-25 VB-50 VB-100
F
MIN6
a
p-AKT/AKT relative expression level
0.0 0.5 1. 1.5
VB-0 VB-12.5 VB-25 VB-50 VB-100

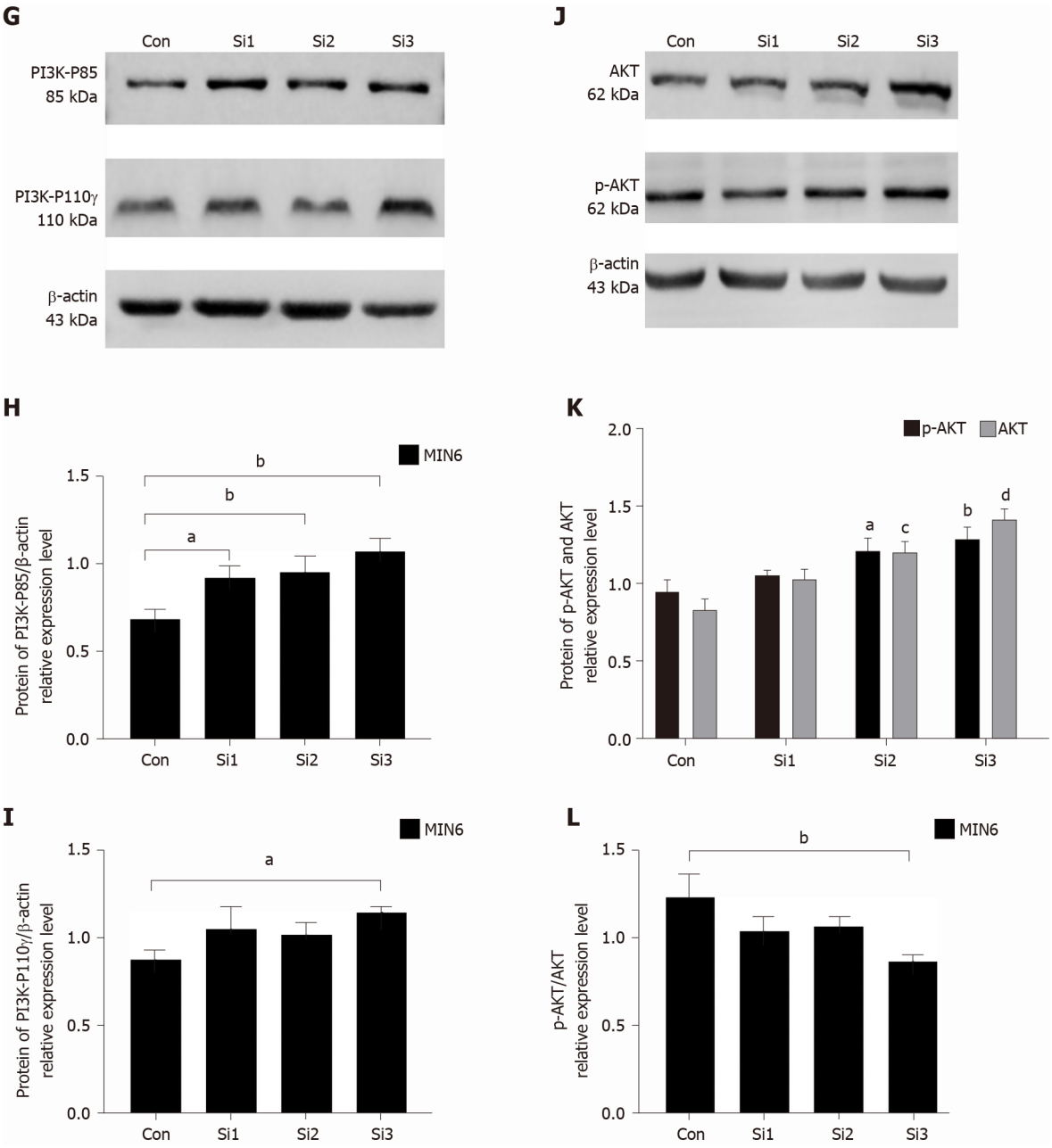


Figure 3 Exogenous vascular endothelial growth factor B inhibits the expression of PI3K-AKT pathway proteins in MIN6 cells, and knockdown of vascular endothelial growth factor B promotes the expression of these pathway proteins. A-C: Western blot analysis revealing the relative protein expression of phosphatidylinositol 3-kinase (PI3K)-P85 and PI3K-P110γ in cells treated with exogenous vascular endothelial growth factor B (VEGF-B, VB) and corresponding statistical data. $^{a}P < 0.05$ *vs* VB-0, $^{b}P < 0.01$ *vs* VB-0; D and E: Western blot analysis indicating the relative protein expression of phosphorylated (p)-serine/threonine kinase (AKT) and AKT in cells treated with exogenous VEGF-B and corresponding statistical data. $^{a}P < 0.05$ *vs* VB-0 about p-AKT; F: The effect of exogenous VEGF-B protein stimulation on AKT phosphorylation. $^{a}P < 0.05$ *vs* VB-0; G-I: Western blot analysis revealing the relative protein expression of PI3K-P85 and PI3K-P110γ in cells transfected with VEGF-B small interfering RNA (siRNA) and corresponding statistical data. $^{a}P < 0.05$ *vs* Control, $^{b}P < 0.01$ *vs* Control; J and K: Western blot analysis indicating the relative protein expression of p-AKT and AKT in cells transfected with VEGF-B siRNA and corresponding statistical data. $^{a}P < 0.05$ *vs* Control, $^{b}P < 0.01$ *vs* Control for p-AKT levels; $^{c}P < 0.01$ *vs* Control, $^{d}P < 0.001$ *vs* Control for AKT levels; L: Effect of VEGF-B knockdown on AKT phosphorylation. $^{b}P < 0.01$ *vs* Con *vs* Control. Student's *t*-test was performed. PI3K: Phosphatidylinositol 3-kinase; AKT: Serine/threonine kinase; p-AKT: Phosphorylated-serine/threonine kinase; VB: Vascular endothelial growth factor B; Con: Control; Si: Small interfering RNA; MIN6: MIN6 cell.

and p-AKT in the VB-12.5 group were higher than those in the VB-0 group, while decreased in the other three groups compared to those in the VB-0 group. The expression levels of p-AKT in the VB-50 and VB-100 groups were obviously lower than that of the VB-0 group ($P < 0.05$). The p-AKT/AKT ratio in MIN6 cells treated with VEGF-B protein was lower than that of VB-0, and the VB-50 group had the lowest ratio ($P < 0.05$) (Figure 3D-F).

MIN6 cells with siRNA knockdown of VEGF-B showed higher expression levels of

PI3K-p85 and PI3K-p110γ than the Con cells at 48 h after transfection ($P < 0.01$ and $P < 0.05$, respectively) (Figure 3G-I).

After siRNA knockdown of VEGF-B in MIN6 cells for 48 h, there were higher expression levels of AKT and p-AKT than those in the Con cells ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). The p-AKT/AKT ratio was significantly lower in the VEGF-B-knockdown cells than in Con cells ($P < 0.01$) (Figure 3J-L).

Effects of VEGF-B on cAMP and Ca^{2+} levels and PLCγ1 protein expression in MIN6 cells

When the glucose concentration was 5.5 mmol/L, MIN6 cells stimulated with 50 and 100 ng/mL exogenous VEGF-B showed significantly lower cAMP levels than the VB-0 cells ($P < 0.05$ for both). When the glucose concentration was 25 mmol/L, the cAMP levels of all the VEGF-B-treated cell groups decreased compared with those of the VB-0 group, and the decrease in cAMP level in the VB-100 group was significantly different ($P < 0.01$) (Figure 4A).

At a glucose concentration of 5.5 mmol/L, MIN6 cells stimulated with exogenous VEGF-B showed decreased Ca^{2+} levels compared with those of the control cells; the decreases in the VB-50 and VB-100 groups were significantly reduced ($P < 0.01$) (Figure 4C).

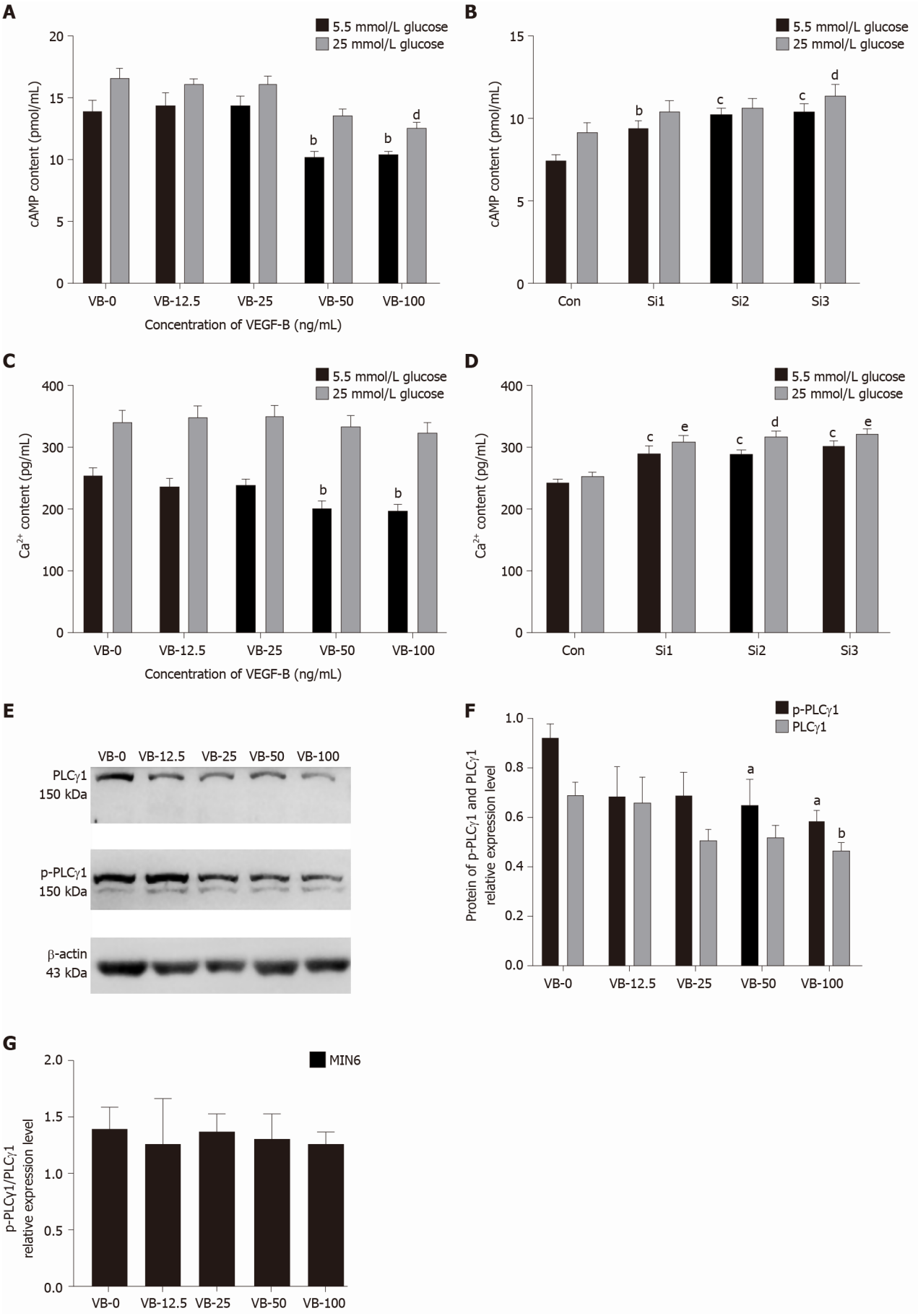
After siRNA knockdown of VEGF-B in MIN6 cells, the cells were cultured in 5.5 mmol/L or 25 mmol/L glucose for 24 h. Compared with those of the Con groups, intracellular Ca^{2+} and cAMP levels were increased ($P < 0.05$, $P < 0.01$, and $P < 0.001$) (Figure 4B and D).

Compared with those of the VB-0 group, the PLCγ1 and p-PLCγ1 protein expression levels and the p-PLCγ1/PLCγ1 ratio in the VEGF-B-stimulated groups were all reduced; the reductions in the VB-50 and VB-100 groups were statistically different compared with those of the VB-0 treatment ($P < 0.05$) (Figure 4E-G).

MIN6 cells with siRNA-mediated knockdown of VEGF-B showed increasing protein expression of PLCγ1 and p-PLCγ1 compared to the control cells at 48 h after transfection ($P < 0.05$). However, the p-PLCγ1/PLCγ1 ratios were not statistically different between the VEGF-B-knockdown and Con groups (Figure 4H-J).

DISCUSSION

Seven VEGF family members have been identified in mammals: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor (PIGF)[9,10]. The binding of members of the VEGF family with three tyrosine kinase receptors, namely, VEGFR1, VEGFR2, and VEGFR3, results in downstream biological activity. In addition, neuropilins, such as NRP1 and NRP2, can cooperate with VEGFR[11-13]. VEGF-B, a recently discovered factor in the VEGF family, is a glycoprotein composed of homodimers that are covalently linked by disulfide bonds[14]. VEGF-B is expressed in tissues with high metabolic activity, such as heart, skeletal muscle, and brown fat, but unlike other VEGF family members, VEGF-B does not significantly promote angiogenesis under physiological conditions. Once apoptosis and other degenerative pathological changes occur, VEGF-B protects cells from apoptosis and exerts a prosurvival effect[8,15]. By contrast, if there is a high concentration of growth-promoting factors, VEGF-B can inhibit growth and prevent excessive growth. VEGF-B acts to maintain the normal state of the body; for example, VEGF-B protects the nervous system while playing a protective role in blood vessels, and it does not cause cardiovascular, cerebrovascular, or nerve damage, showing superior safety as a drug target[16-18]. In addition, VEGF-B can also act as an antioxidant to protect tissues and cells from damage induced by oxidative stress[19,20]. In pathological conditions such as diabetes, obesity, and cardiovascular disease, VEGF-B regulates lipid accumulation, but its mechanism of action is still unclear in these processes[21]. In 2010, Hagberg *et al*[22] first proposed in "Nature" that VEGF-B affects the abnormal metabolism of FAs in endodermal cells. Subsequently, the team discovered that abnormal lipid deposition in peripheral tissues can impair insulin sensitivity and affect glucose uptake[2]. In 2016, the Mehlem *et al*[3] further confirmed that inactivation of VEGF-B could improve insulin resistance, and high levels of VEGF-B could cause insulin resistance and blood glucose deterioration. In the same year, Robciuc *et al*[5] performed a study on obese and insulin-resistant mice and showed that VEGF-B gene transfer and endothelial VEGFR1 conduction blockade led to weight loss and reduced metabolic-related complications. These findings reveal the therapeutic role of the VEGF-B/VEGFR1 pathway in obesity and T2D. VEGF-B may be a considerable factor in the influence of insulin secretion


A
5.5 mmol/L glucose
25 mmol/L glucose
cAMP content (pmol/mL)
VB-0
VB-12.5
VB-25
VB-50
VB-100
Concentration of VEGF-B (ng/mL)
B
5.5 mmol/L glucose
25 mmol/L glucose
cAMP content (pmol/mL)
Con
Si1
Si2
Si3
C
5.5 mmol/L glucose
25 mmol/L glucose
Ca2+ content (pg/mL)
VB-0
VB-12.5
VB-25
VB-50
VB-100
Concentration of VEGF-B (ng/mL)
D
5.5 mmol/L glucose
25 mmol/L glucose
Ca2+ content (pg/mL)
Con
Si1
Si2
Si3
E
VB-0
VB-12.5
VB-25
VB-50
VB-100
PLCγ1
150 kDa
p-PLCγ1
150 kDa
β-actin
43 kDa
F
p-PLCγ1
PLCγ1
Protein of p-PLCγ1 and PLCγ1
relative expression level
VB-0
VB-12.5
VB-25
VB-50
VB-100
G
MIN6
p-PLCγ1/PLCγ1
relative expression level
VB-0
VB-12.5
VB-25
VB-50
VB-100

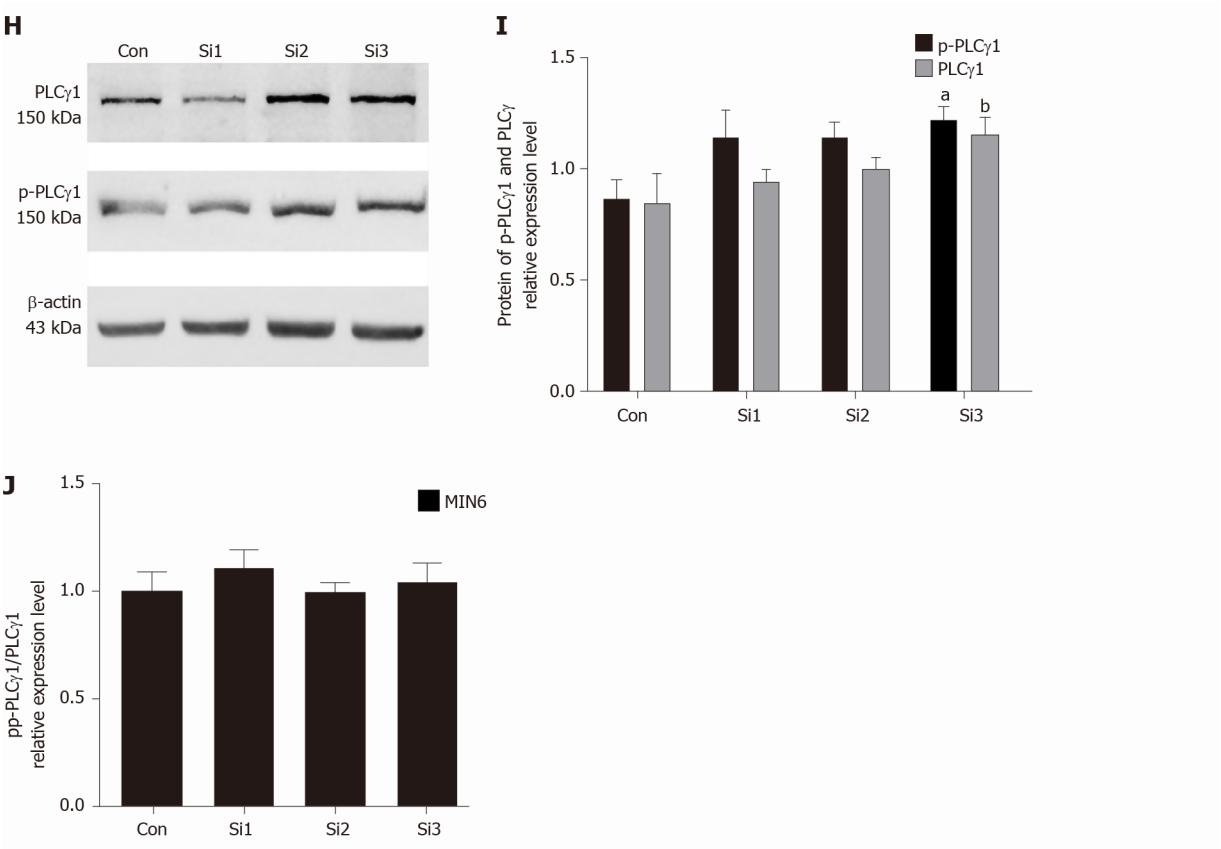


Figure 4 Vascular endothelial growth factor B inhibits Ca^{2+} and cyclic adenosine monophosphate levels and phospholipase C gamma 1 protein expression in MIN6 cells. A: Exogenous vascular endothelial growth factor B (VEGF-B, VB) protein reduces cyclic adenosine monophosphate (cAMP) levels in MIN6 cells. $^{b}P < 0.01$ *vs* VB-0 in the presence of 5.5 mmol/L glucose; $^{d}P < 0.01$ *vs* VB-0 in the presence of 25 mmol/L glucose; B: cAMP levels in MIN6 cells increase after VEGF-B is knocked down. $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* Control in the presence of 5.5 mmol/L glucose; $^{d}P < 0.05$ *vs* Control in the presence of 25 mmol/L glucose; C: Exogenous VEGF-B protein reduces Ca^{2+} levels in MIN6 cells. $^{b}P < 0.01$ *vs* VB-0 in the presence of 5.5 mmol/L glucose; D: Ca^{2+} levels in MIN6 cells increase after VEGF-B is knocked down. $^{c}P < 0.001$ *vs* Con in the presence of 5.5 mmol/L glucose; $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* Con in the presence of 25 mmol/L glucose; E and F: Western blot analysis revealing the relative protein expression of phospholipase C gamma 1 (PLCγ1) and phosphorylated (p)-PLCγ1 in cells stimulated with exogenous VEGF-B and corresponding statistical data. $^{a}P < 0.05$ *vs* VB-0 for P-PLCγ1; $^{b}P < 0.05$ *vs* VB-0 for PLCγ1; G: The effect of exogenous VEGF-B protein stimulation on PLCγ1 phosphorylation; H and I: Western blot analysis revealing the relative protein expression of PLCγ1 and p-PLCγ1 in cells transfected with VEGF-B siRNA and corresponding statistical data. $^{a}P < 0.05$ *vs* Control for p-PLCγ1, $^{b}P < 0.05$ *vs* Control for PLCγ1; J: Effect of VEGF-B knockdown on PLCγ1 phosphorylation. Student's *t*-test was performed. VEGF-B: Vascular endothelial growth factor B; VB: VEGF-B; cAMP: Cyclic adenosine monophosphate; Con: Control; Si: Small interfering RNA; MIN6: MIN6 cell; PLCγ1: Phospholipase C gamma 1; p-PLCγ1: Phosphorylated phospholipase C gamma 1.

and is expected to become an important factor for maintaining blood glucose balance and affecting insulin secretion[21,23].

In 2020, Chen *et al*[24] published a study on a mouse model with fat-specific VEGF-B inhibition to better elucidate the role of VEGF-B in fat development and energy metabolism. After inhibiting VEGF-B, the mice had larger bodies, more white adipose tissue appeared in the mice, and the morphology and function of brown fat changed to those of white fat. It was proposed that VEGF-B expressed in fat is a function of fat development and a main regulator of fat function[24]. In a study of a mouse model of pancreatic β-cell-specific VEGF-B deficiency, Ning *et al*[4] found that the specific deletion of VEGF-B can increase insulin secretion but does not have much effect on the glucose tolerance or insulin sensitivity of mice. Moreover, the study also showed that VEGF-B-deficient mice fed an HFD exhibited no alterations in glucose homeostasis, and pancreatic islet lipid uptake was not altered[4]. In addition to the study of model mice, in 2017, Wu *et al*[25] found that the plasma VEGF-B level of T2D patients was significantly higher than that of subjects with normal glucose tolerance, and VEGF-B was related to the first stage of glucose-stimulated β-cell insulin secretion, indicating that VEGF-B may be related to abnormal glucose metabolism.

Dijkstra *et al*[26] hold a different view from Hagberg's team. Researchers believe that VEGF-B has little effect on changes in lipid metabolism in a mouse model of HFD-induced diabetes. In 2014, Sun *et al*[27] also showed that VEGF-B (-/-) model mice did not show much change in body weight or glucose metabolism upon HFD consumption compared with littermate control mice. In addition, Sun *et al*[27] performed a

comparative study between T2D patients and healthy adults and showed that the plasma concentration of VEGF-B was not much different in the two groups.

Based on studies of the phenotypes of animal models of diabetes and humans with diabetes, the mechanism by which VEGF-B affects glucose and lipid metabolism is still controversial. Research on VEGF-B involvement in insulin sensitivity has attracted much attention in recent years. The MIN6 cell line was derived from mouse insulinoma and is very similar in function to pancreatic islets in mice. This cell line can be used as a model to simulate β-cell functions and study insulin secretion *in vitro*. We added VEGF-B to subcultured MIN6 cells and observed changes in cell proliferation and apoptosis. After stimulation of MIN6 cells with 50 ng/mL and 100 ng/mL VEGF-B for 24 h, cell proliferation was higher than that of cells without VEGF-B treatment, and there was no significant change in apoptotic cells. Although studies have found that VEGF-B affects the proliferation of cultured MIN6 cells, the effect of VEGF-B on insulin secretion in MIN6 cells needs to be further examined.

Insulin secretion and its regulation are important mechanisms for maintaining glucose homeostasis in the body. Insufficient insulin secretion can lead to non-insulin-dependent diabetes. Insulin is encapsulated in vesicles with dense cores, and pancreatic β-cells regulate insulin secretion by regulating the exocytosis and secretion of these vesicles. Intracellular Ca^{2+} is an important factor affecting insulin secretion; pancreatic islet β-cells change the intracellular Ca^{2+} concentration mainly through ATP-sensitive calcium channels on the plasma membrane and intracellular calcium store activities, thereby regulating insulin secretion[28,29]. Under physiological conditions, an increase in the Ca^{2+} concentration can directly trigger the secretion of insulin by pancreatic β-cells. When the glucose concentration is higher than the stimulation threshold, ATP production in β-cells increases through metabolic processes[30], thereby closing the K-ATP channel on the cell membrane, depolarizing the membrane, and inducing action potentials to open L-type Ca^{2+} channels; this results in extracellular Ca^{2+} influx and an increase in intracellular Ca^{2+}, which further activates the release of intracellular Ca^{2+} pools and finally activates insulin vesicles to release a large amount of insulin *via* exocytosis to accelerate the absorption and utilization of glucose by all human tissues (except brain)[31-35].

In addition to causing changes in Ca^{2+}, glucose also increases the cAMP levels in pancreatic β-cells. Glucose in pancreatic β-cells can cause rapid changes in intracellular Ca^{2+} and cAMP concentrations. These effects are independent of glucose metabolism and are mediated by glucose-sensitive receptors[36]. Increased cAMP in the cytoplasm is induced by a high concentration of glucose and is secondary to the increase in Ca^{2+}; that is, the increase in Ca^{2+} increases the production of cAMP[37,38]. On the other hand, cAMP can also increase the sensitivity of mouse pancreatic β-cell exocytosis to Ca^{2+}[39]. In our study, we found that after 24 h of glucose induction, MIN6 cells treated with 50 ng/mL and 100 ng/mL VEGF-B exhibited decreased insulin secretion. By contrast, after siRNA-mediated knockdown of VEGF-B for 48 h, MIN6 cells showed increased insulin secretion. This was consistent with the study of Ning *et al*[4], who found that in a VEGF-B-deficient mouse model, the specific deletion of VEGF-B in β-cells could upregulate insulin gene expression and increase insulin secretion. Our research also showed that after 24 h of glucose induction, stimulation with 50 ng/mL and 100 ng/mL exogenous VEGF-B can cause decreases in the intracellular Ca^{2+} and cAMP concentrations in MIN6 cells. After siRNA-mediated knockdown of VEGF-B for 48 h, the Ca^{2+} and cAMP concentrations in MIN6 cells were higher than those in Con cells. The results of the study showed that after exogenous VEGF-B stimulation or siRNA-mediated knockdown of VEGF-B in MIN6 cells, the changes in insulin secretion and intracellular Ca^{2+} and cAMP levels were consistent. We found that VEGF-B is involved in insulin secretion in MIN6 cells and that VEGF-B may affect insulin secretion by changing the intracellular Ca^{2+} concentration and cAMP level. In the same year, Robciuc *et al*[5] studied obese and insulin-resistant mice and found that VEGF-B gene transfer and VEGFR1 gene deletion led to weight loss and reduced metabolic complications. These findings reveal the therapeutic role of the VEGF-B/VEGFR1 pathway in obesity and T2D.

As a glycoprotein, VEGF-B mainly acts by binding to the membrane receptors VEGFR1 and NRP1. The full-length *VEGFR1* gene is 7680 bp, which encodes 1338 amino acids. The seven Ig-like domains in the extracellular region are responsible for binding the VEGF ligand and promoting angiogenesis[40]. VEGFR1 is mainly expressed in endothelial cells, blastoderm trophoblasts, renal interstitial cells, and other cells[41]. The functions and properties of VEGFR1 can be determined according to the developmental stages of different cells in different animals. VEGFR1 can bind to not only VEGF-A but also PLGF and VEGF-B, but neither PIGF nor VEGF-B can bind VEGFR2. NRP1 is a non-tyrosine kinase single-pass transmembrane glycoprotein

approximately 130-140 KD in size that was originally discovered as a receptor for nerve guidance factors (semaphorin 3A, Sema3A) involved in regulating nerve cell guidance and axon growth. NRP1 is composed of three parts: An intracellular domain, a transmembrane domain, and an extracellular domain (a1/a2, b1/b2, and c domain). The a1/a2 domain, also known as the CUB domain, mainly binds to Sema3A; the b1/b2 domain is mainly related to the binding of VEGF family members and mediating angiogenesis; and the c domain mainly mediates the dimerization of NRP1 and is closely related to its signal transduction[42]. In 2016, Robciuc *et al*[5] reported on cellular metabolism and stated that increasing VEGF-B levels do not affect the weight of mice but can improve glucose metabolism, enhance insulin sensitivity, reduce inflammation, and significantly improve the metabolic health of obese mice. Moreover, deletion of the *VEGFR1* gene in endothelial cells enhances the effect of VEGF-B, activates thermogenesis in subcutaneous fat tissue, increases the basal metabolic rate, and prevents obesity and related metabolic complications caused by diet.

Robciuc *et al*[5] believed that VEGF-B competes with VEGF-A to bind VEGFR1 and promotes the binding of VEGF-A and VEGFR2 to increase angiogenesis and perfusion of adipose tissues to improve insulin secretion and signal transduction through vascular remodeling. The binding of VEGF-B and VEGFR1 activates the VEGF/VEGFR2 pathway and increases capillary density in adipose tissue, tissue blood circulation, and signal transduction.

VEGFR1 was the first VEGF receptor discovered. It has a higher affinity for the VEGF family of ligands than VEGFR2 but has weak tyrosine kinase activity, and the expression level of VEGFR1 on endothelial cells is lower than that of VEGFR2. Many biological functions of VEGF are mainly mediated by VEGFR2. VEGFR1 also has a synergistic effect with VEGFR2, and the two receptors can jointly regulate multiple signal transduction pathways[43]. For many years, VEGFR1 has been considered to have only modified auxiliary functions. However, in recent years, scientific exploration has found that the expression of VEGFR1 is increased in many pathological conditions, and this receptor can mediate some biological functions, such as angiogenesis in ischemic diseases, remodeling after vascular injury, atherosclerosis, and certain inflammatory diseases[44,45]. Studies have shown that VEGFR1 can also bind to proteins such as PI3K, PLCγ, Grb2, SHP2, and Nck, but the downstream signal transduction pathways involved are unclear[17,46]. In our study, we found that after 24 h of treatment with 50 ng/mL or 100 ng/mL VEGF-B, the protein expression level of VEGFR1 in MIN6 cells was significantly increased, and the protein expression level of NRP1 also increased. We further observed the effect of VEGF-B on PLCγ1, PI3K, AKT, and other molecules downstream of the VEGF-B/VEGFR1 signaling pathway in this study. Our study found that exogenous VEGF-B protein reduced the expression of the PI3K kinase isoforms p85 and p110γ in MIN6 cells, and after knocking down VEGF-B, the expression of p85 and p110γ increased significantly. The study also showed that exogenous VEGF-B simultaneously reduced the expression of AKT and P-AKT in MIN6 cells. Upon knockdown of VEGF-B, the expression of these proteins in MIN6 cells also increased significantly.

A large number of studies have shown that the PI3K-AKT signaling pathway is related to cell proliferation, for instance, lactoglobulin-mediated promotion of C2C12 cell proliferation[47]. By inhibiting the activation of PI3K/AKT, the resultant downregulation of LRRC8A (leucine-rich repeat-containing 8A) activity can inhibit the angiotensin II-induced proliferation of cerebral vascular smooth muscle cells[48]. FER1L4 (Fer-1-like family member 4) inhibits the proliferation and metastasis of lung cancer cells by regulating the PI3K/AKT pathway[49]. Prucalopride inhibits the proliferation, invasion, and migration of lung cancer cells by inhibiting the PI3K/AKT pathway[50].

CCK-8 analysis showed that in MIN6 cells with VEGF-B knockdown, exogenous VEGF-B administration induced MIN6 cell proliferation. We hypothesize that the MIN6 cell proliferation observed in this study was not necessarily related to the PI3K/AKT pathway. The results of Western blot indicated that after VEGF-B intervention, the protein expression of PI3K, P-PI3K, AKT, and P-AKT was consistent with the trend of insulin secretion. When exogenous VEGF-B was used to stimulate MIN6 cells, the secretion of insulin was reduced, and the expression of PI3K/AKT pathway related proteins was also reduced. After siRNA-mediated knockdown of VEGF-B, the amount of insulin secretion and the protein expression of PI3K, P-PI3K, AKT, and P-AKT increased.

PI3K is a kinase that specifically catalyzes phosphatidylinositol. In glucose homeostasis, insulin can regulate β-cell function and insulin secretion through the PI3K/AKT pathway[51,52]. MacDonald *et al*[53] showed that the p110γ isoform of PI3K kinase can positively regulate glucose-stimulated insulin secretion. Inhibition of the

PI3K catalytic subunit p110γ can significantly reduce insulin secretion and exocytosis induced by depolarization and directly eliminate exocytosis caused by Ca^{2+} influx, thereby inhibiting insulin secretion[54]. Our research results are consistent with these findings.

Phosphatidylinositol is very important in the regulation of vesicle transport during the release of insulin secretory vesicles caused by a large influx of Ca^{2+}[55]. In pancreatic cells, glucose can activate PLC in the cytoplasm, and the influx of calcium also activates PLC, causing the production of inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 in turn induces the endoplasmic reticulum to release intracellular Ca^{2+}, and DAG induces the transport of protein kinase C to the plasma membrane and promotes insulin secretion[56,57]. Fiume *et al*[58] further proved that insulin secretion in MIN6 cells was regulated by PLC. The inhibition of PLC leads to impaired insulin secretion, and among the isoforms of PLC, PLCγ1 has the greatest impact on insulin secretion[59]. Therefore, we explored the expression changes of PLCγ1 and P-PLCγ1 as it relates to the mechanism by which VEGF-B affects insulin expression and found that after stimulation with exogenous VEGF-B, the protein expression of PLCγ1 and p-PLCγ1 in MIN6 cells decreased. After knocking down VEGF-B in these cells, the protein expression of PLCγ1 and p-PLCγ1 increased. We hypothesize that the inhibition of insulin secretion by VEGF-B is also related to the PLC signaling pathway.

Our research indicated that the effects of VEGF-B on insulin secretion and Ca^{2+} and cAMP levels in MIN6 cells were mediated by the PI3K/AKT pathway. First, stimulation with exogenous VEGF-B decreased insulin secretion from MIN6 cells, and the levels of Ca^{2+} and cAMP related to insulin secretion in these cells also decreased. Moreover, the changes in the expression of key proteins in insulin secretion signaling pathways, such as PLCγ1 and PI3K/AKT, were consistent with the changes in insulin levels. In addition, the increased insulin secretion of MIN6 cells with downregulated VEGF-B expression was confirmed by the direct transfection of VEGF-B siRNA. The changes in the levels of Ca^{2+} and cAMP and the expression of PLCγ1, PI3K/AKT and other proteins were also consistent with the changes in insulin levels. These results confirm our hypothesis that VEGF-B affects insulin secretion by MIN6 cells through the PI3K/AKT signaling pathway (Figure 5), which may provide an explanation for the involvement of VEGF-B in the pathogenesis of T2D. However, whether VEGF-B directly functions by binding VEGFR1 or competing with VEGF-A to promote the binding of VEGF-A and VEGFR2 to activate downstream signaling pathways remains to be further studied[5]. In addition, these findings encourage us to continue to explore the effect of VEGF-B on insulin secretion by regulating the expression level of VEGF-B in mice.

CONCLUSION

The results of this study show that VEGF-B can regulate insulin secretion by modulating the levels of Ca^{2+} and cAMP. VEGF-B involvement in insulin secretion is related to the expression of PLCγ1, PI3K, AKT, and other signaling proteins. These results provide theoretical support and an experimental basis for the study of VEGF-B in the pathogenesis of T2D.

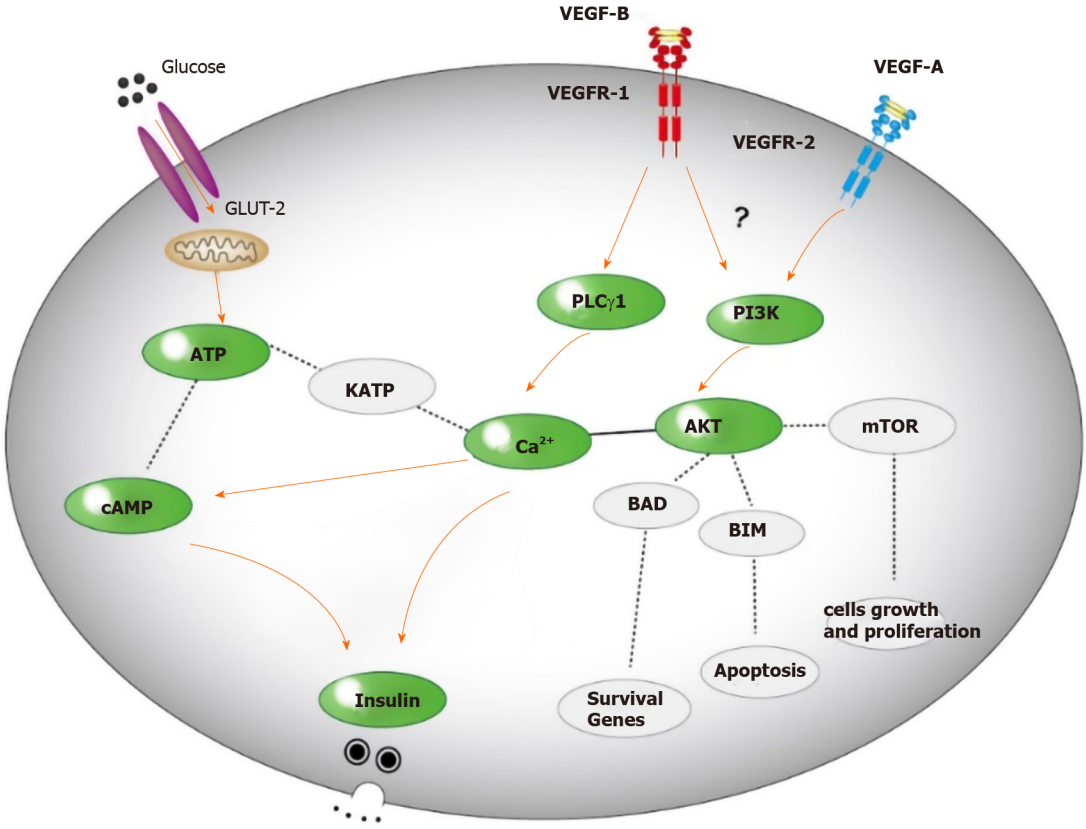


Figure 5 Possible mechanism for VEGF-B to regulate insulin secretion.

ARTICLE HIGHLIGHTS

Research background

Type 2 diabetes (T2D), which is characterized by defective pancreatic β-cell function or insulin resistance leading to insufficient insulin secretion and increased blood sugar, has elicited worldwide public health concerns. Despite the knowledge available on T2D, the mechanism of insulin secretion is still unclear. In this study, we studied the mechanism by which vascular endothelial growth factor B (VEGF-B) affects the insulin secretion signaling pathway in MIN6 cells and explored the role of VEGF-B in blood glucose regulation.

Research motivation

We explored the role of VEGF-B in the insulin secretion signaling pathway and provided mechanistic insights into the occurrence and development of insulin secretion and T2D.

Research objectives

Our aim was to explore the mechanism of insulin secretion, study the effect of VEGF-B on insulin secretion, and provide a new strategy to prevent the progression of T2D.

Research methods

This study was performed with *in vitro* cultures of MIN6 cells. By studying the effect of VEGF-B on insulin secretion in MIN6 cells and detecting the levels of Ca^{2+} and cyclic adenosine monophosphate (cAMP) in the insulin secretion signaling pathway and the expression of key proteins in the PI3K-AKT (phosphatidylinositol 3-kinase-serine/threonine kinase) signaling pathway, the effect of VEGF-B on the insulin secretion mechanism was discussed. Statistical analyses were performed using SPSS statistical software (version 22.0).

Research results

In this study, we found that exogenous VEGF-B treatment inhibited the secretion of insulin and simultaneously reduced the levels of Ca^{2+} and cAMP in MIN6 cells. In

MIN6 cells with VEGF-B knockdown, insulin secretion and Ca^{2+} and cAMP levels increased. The effect of VEGF-B on insulin occurred through the PI3K-AKT pathway.

Research conclusions

VEGF-B can inhibit insulin secretion through the PI3K-AKT pathway and may become a new target for the study of T2D. Our study provides new mechanistic insight into insulin secretion.

Research perspectives

This study could provide new insights into the mechanism of insulin secretion and form the foundation for new ideas for the prevention of T2D.

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ORIGINAL ARTICLE

Basic Study

Three-dimensional-arterial spin labeling perfusion correlation with diabetes-associated cognitive dysfunction and vascular endothelial growth factor in type 2 diabetes mellitus rat

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Author contributions: Shao JW designed experiments, performed the literature review, and wrote the draft of the paper; Wang JD and Zou YY helped establish the type 2 diabetes mellitus rat models and assisted in the histopathologic procedures; Li JB scanned the rats; Su W collected the data; He Q and Xiang ST corrected the data; Yang Y also contributed to the writing and editing of the manuscript, which was critically revised and edited by Fang J; All authors approved the final version.

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Abstract

BACKGROUND

Type 2 diabetes mellitus (T2DM) has been strongly associated with an increased risk of developing cognitive dysfunction and dementia. The mechanisms of diabetes-associated cognitive dysfunction (DACD) have not been fully elucidated to date. Some studies proved lower cerebral blood flow (CBF) in the hippocampus was associated with poor executive function and memory in T2DM. Increasing evidence showed that diabetes leads to abnormal vascular endothelial growth factor (VEGF) expression and CBF changes in humans and animal models. In this study, we hypothesized that DACD was correlated with CBF alteration as measured by three-dimensional (3D) arterial spin labeling (3D-ASL) and VEGF

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Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals (Kunming Medical University Institutional Review Board, Approval No. kmmu 2020410).

Conflict-of-interest statement: No potential conflicts of interest.

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expression in the hippocampus.

AIM

To assess the correlation between CBF (measured by 3D-ASL and VEGF expression) and DACD in a rat model of T2DM.

METHODS

Forty Sprague-Dawley male rats were divided into control and T2DM groups. The T2DM group was established by feeding rats a high-fat diet and glucose to induce impaired glucose tolerance and then injecting them with streptozotocin to induce T2DM. Cognitive function was assessed using the Morris water maze experiment. The CBF changes were measured by 3D-ASL magnetic resonance imaging. VEGF expression was determined using immunofluorescence.

RESULTS

The escape latency time significantly reduced 15 wk after streptozotocin injection in the T2DM group. The total distance traveled was longer in the T2DM group; also, the platform was crossed fewer times. The percentage of distance in the target zone significantly decreased. CBF decreased in the bilateral hippocampus in the T2DM group. No difference was found between the right CBF value and the left CBF value in the T2DM group. The VEGF expression level in the hippocampus was lower in the T2DM group and correlated with the CBF value. The escape latency negatively correlated with the CBF value. The number of rats crossing the platform positively correlated with the CBF value.

CONCLUSION

Low CBF in the hippocampus and decreased VEGF expression might be crucial in DACD. CBF measured by 3D-ASL might serve as a noninvasive imaging biomarker for cognitive impairment associated with T2DM.

Key Words: Diabetes-associated cognitive dysfunction; Diabetes mellitus; Type 2; Perfusion imaging; Receptors; Vascular endothelial growth factor; Hippocampus; Three-dimensional pseudo-continuous arterial spin labeling

Core Tip: This study aimed to assess the correlation between cerebral blood flow measured by three-dimensional arterial spin labeling, vascular endothelial growth factor expression, and diabetes-associated cognitive dysfunction in a rat model of type 2 diabetes (T2D). Our results showed low cerebral blood flow in the hippocampus and decreased vascular endothelial growth factor expression might be crucial in diabetes-associated cognitive dysfunction. Cerebral blood flow measured by three-dimensional arterial spin labeling might serve as a noninvasive imaging biomarker for cognitive impairment associated with T2D. This study would help in the early detection of diabetes-associated cognitive dysfunction and guide treatment.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is an endocrine and chronic metabolic disorder characterized by insulin resistance and insulin deficiency caused by pancreatic b-cell dysfunction[1]. The number of people with diagnosed or undiagnosed diabetes, aged 20-64 years, was 351.7 million in 2019, and T2DM accounted for more than 90% of all

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Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
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cases. Approximately 116.4 million adults aged 20-79 years have diabetes in China, which ranked first worldwide. The United States has the third most affected adults (31.0 million)[2]. T2DM is one of the largest public health problems and represents a global public health challenge due to a gradual increase in its incidence.

The major complications of T2DM are stroke, dementia, and depression. It is associated with a 1.5 times increased risk of dementia[3]. Dementia is a complex condition marked by diminished cognitive performance (*i.e.* language, memory, visuospatial, and executive functions), affecting the quality of patients' everyday lives[4,5]. T2DM has been strongly associated with an increased risk of developing Alzheimer's disease and vascular dementia, accounting for 95% of all dementia cases[6,7]. The mechanisms of diabetes-associated cognitive dysfunction (DACD) have not been fully elucidated to date. The white matter disease of vascular origin, inflammation, cerebral insulin resistance, axonal loss, and vascular endothelial dysfunction may be the mechanisms underlying the dementia risk in diabetes[8-10].

Vascular endothelial growth factor (VEGF; also known as VEGF-A) is involved in microvascular structure and function and the development of axon branching[11,12]. Many studies have shown that enhancing VEGF signaling or VEGF restoration can ameliorate cognitive function[13,14]. Most of these studies have focused on the relationship between serum VEGF level and cognitive performance. Few studies explored how the differences in VEGF expression in the hippocampus influenced cognitive ability.

The hippocampus is a part of the limbic system. It has multimodal roles in the integration of information from short-term memory with that from long-term memory[15-17]. Perception and memory impairment have resulted from damage to the hippocampus, in turn contributing to cognitive dysfunction[18]. Most previous brain studies on T2DM have revealed the whole brain and hippocampal macrostructural changes in diabetes using magnetic resonance imaging (MRI), including cerebral perfusion changes, atrophy, and decreased white matter fiber connection linked to poor cognitive performance[19-22]. Previous findings showed that lower cerebral blood flow (CBF) in the hippocampus was associated with poor executive function and memory in T2DM[23,24].

Three-dimensional pseudo-continuous arterial spin labeling (3D-ASL) provides an intrinsically high signal-to-noise ratio and precise detection of CBF[25]. It is noninvasive MRI technique that involves no contrast agent or ionizing radiation. In this study, the 3D-ASL approach was applied to analyze CBF in the hippocampus. Despite increasing evidence that diabetes leads to abnormal VEGF expression and CBF changes in humans and animal models, no study explored whether VEGF signaling in the hippocampus in the T2DM animal model was related to CBF measured by 3D-ASL or aimed to assess the relationship between VEGF expression in the hippocampus and DACD, which became the objectives of the present study.

Sprague-Dawley rats have been used as a model of type 2 diabetes in many experiments. In this study, T2DM Sprague-Dawley rats were used to conduct the experiments. The study found that diabetes led to a reduction of VEGF expression in the hippocampus of rats with T2DM. Decreased VEGF expression in the hippocampus and reduction of hippocampal CBF positively correlated with poor cognitive function.

MATERIALS AND METHODS

Animals

The experimental procedures were carried out according to the National Institutes of Health guidelines for the care of experimental animals with approval from the institutional animal ethics committee of the Kunming Medical University. All efforts were made to minimize suffering or animal discomfort and the number of animals used.

An online power and sample size calculator (Power and Sample Size.com) was used to calculate the sample size. Forty specific-pathogen-free grade Sprague-Dawley male rats (aged 4-5 wk and weighing 200-220 g) from the Experimental Animal Centre of Kunming Medical University (certificate o. SCXK 2015-0002; Kunming, China) were used in this study. Before initiating the protocol, all animals were acclimatized in the institutional animal house for 2 wk. The animals were randomly divided into two groups: normal control (n = 20) and T2DM (n = 20). Weight and fasting blood glucose (FBG) were measured before the beginning of the experimental procedure. All animals were allowed free access to water with a normal day/night cycle (12 h/12 h), a relative humidity of 40%-50%, and a temperature of 20-25 °C. The normal control group was

fed standard chow, whereas the T2DM group was kept on a high-fat diet (41% carbohydrate, 24% protein, and 24% fat; 4.73 kcal/g; Beijing HFK Bioscience Co., Ltd. Beijing, China) for 3 wk (certificate no. SCXK 2019-0008; Beijing, China).

Streptozotocin (STZ) was purchased from Sigma (MO, United States). After overnight fasting, STZ was administered by intraperitoneal injections on the first, third, and fifth days in the first round, which was repeated with the same dose of STZ on the 21st, 23rd, and 25th days in the second round[26]. The elevated glucose levels in T2DM were evaluated on day 7 (24 h after the last administration). The blood glucose level was measured using an Accu-Chek glucometer (Roche, Mannheim, Germany) in tail-tip blood samples from overnight fasted animals. The rats with FBG > 16.7 mmol/L were considered as the T2DM model[27].

Cognitive behavioral testing

The spatial learning and reference memory of each rat were tested in a Morris water maze (MWM) after the model was successfully established (15 wk after STZ injection). MWM testing was conducted for 6 d, including place navigation for 5 d and spatial probe for 1 d[28]. All behavioral tests were performed under controlled environmental conditions; the water temperature was kept at 22 °C with silence and dim illumination[29]. MWM testing was conducted in a black circular pool, 2 m in diameter and 0.2 m deep filled with water. The water was made opaque with black ink. The position of the hidden platform was kept constant during all the trials involving spatial navigation, and the amount of water exceeded 1 cm of the platform. Each rat was placed at a fixed starting position in every quadrant in spatial navigation and was allowed to swim until the rat reached the platform. The swimming time was 60 s. If it exceeded this time, the rat was guided to the platform. In this procedure, the escape latency and traveled distance were recorded using a video tracking system (ANY-Maze, San Diego Instrument, CA, United States). The interval for each rat between the same tests was 15 min. The platform was removed in the spatial probe test to assess memory consolidation (Figure 1). The swimming trajectory within 60 s and the number of crossings of the platform location were recorded.

MRI scanning

After MWM experiment, the rats were anesthetized with 3.6% chloral hydrate (360 mg/kg, intraperitoneal injection). MRI was performed using a 1.5T scanner (Signa Excite TwinSpeed HDx, GE Healthcare, WI, United States) with a special coil for rats (Shanghai Chenguang Medical Technologies Co., Shanghai, China). The MRI protocol included T1-weighted imaging, T2-weighted imaging, and 3D-ASL. T1-weighted imaging included brain sagittal images, whereas T2-weighted imaging and 3D-ASL included brain coronal images of the hippocampus. MRI scans were acquired in the supine position of the rat. The parameters used were as follows: T1-weighted imaging: time of repetition/time of echo = 260 ms/10.4 ms; scanning time = 138 s; field of view = 100 mm × 100 mm; slice thickness = 2.0 mm; and interslice gap = 0. T2-weighted imaging: time of repetition/time of echo = 2200 ms/85 ms; slice thickness = 1.0 mm; interslice gap = 0; number of excitations = 4; number of slices = 16; matrix size = 224 × 192; field of view = 100 mm × 100 mm; and scanning time = 331 s. 3D-ASL: time of repetition/time of echo = 4132 ms/11 ms; slice thickness = 2.0 mm; no interslice gap; number of excitations = 5; field of view = 80 mm × 80 mm; bandwidth = 62.5 kHz; matrix = 512 point × 12 arms; post-label delay = 1025 ms; and scanning time = 595 s.

3D-ASL image preprocessing

3D-ASL image preprocessing and analysis were implemented using the Advantage Workstation (Advantage Workstation version 4.7, GE Medical Systems, United States), with the Function Tool software package. The threshold was adjusted, and the region of imaging in the bilateral hippocampal area of rats was chosen to measure CBF. Every position was repeated three times and averaged.

Tissue harvesting and immunofluorescence

The rats were sacrificed with an overdose of chloral hydrate. They were given intracardial perfusion of 4% paraformaldehyde, and the brain tissues (hippocampus) of the mice were harvested. The hippocampal tissue samples were frozen in liquid nitrogen and stored at -80 °C prior to proteomic analysis. The hippocampus was dehydrated and then embedded in paraffin. One 10-μm-thick and four 20-μm-thick sections were cut, and 4′, 6-diamidino-2-phenylindole staining was applied for morphological assessment and immunofluorescence staining. All examinations were performed on the bilateral hippocampus. Paraffin sections of the hippocampal tissues

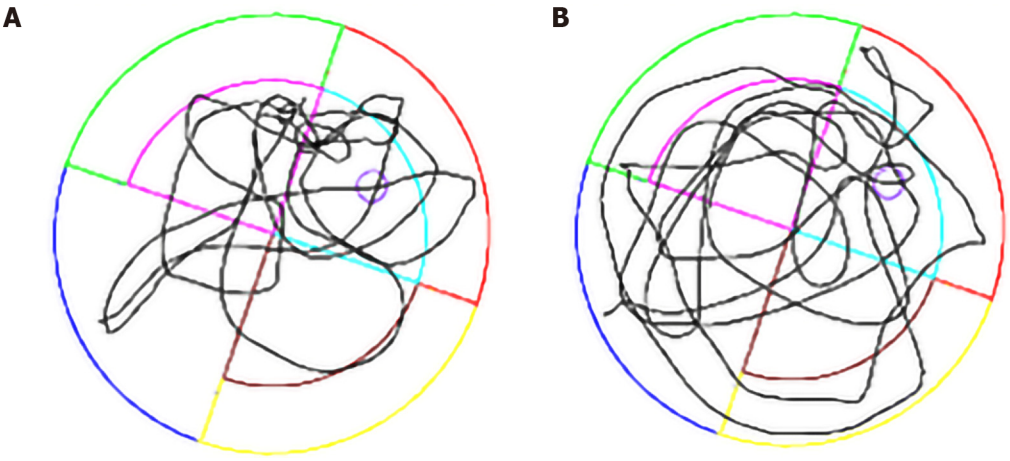

Figure 1 Representative swimming trajectories of rats. A: Control group; B: Comparison with the control group, the swimming trajectory of the rats in the type 2 diabetes mellitus group was more chaotic.

were rehydrated and boiled in ethylene diamine tetraacetic acid buffer for 10 min to induce antigen retrieval. Immunofluorescence of VEGF was performed using commercial kits following the manufacturer's protocols. The sections (40 μm) were incubated with the primary VEGF antibody (Abcam Biotech Co., Ltd., MA, United States). Subsequently, the mixture was incubated with secondary antibodies (Beyotime Biotech Co., Ltd., Nanjing, China). Immunofluorescence-stained sections were observed under a Zeiss Pascal laser scanning confocal microscope (Carl Zeiss International, Jena, Germany).

Quantitative real-time PCR

Total RNA was isolated from the hippocampal tissue of rats in the control and T2DM groups using RNAiso Plus reagent (Takara, Dalian, China) and then reverse transcribed into cDNA using HiScript III RT SuperMix for quantitative PCR (qPCR) (+g DNA wiper) (Vazyme Biotech Co., Ltd., Nanjing, China). Real-time qPCR was performed using miRNA Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) on a BioRad qPCR system with the following primer pair: 5′-TGCATGGTGACTGCTACCTTCTC-3′, 5′-AAATCACAGCAGCCTACCCACTC-3′. The PCR conditions started with a denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94°C for 25 s, annealing at 50.5 °C for 40 s, and extension at 72 °C for 30 s, ending with a final extension step at 72 °C for 5 min. RNA relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method with reduced glyceraldehyde-phosphate dehydrogenase as the control.

Statistical analysis

The data were analyzed using the SPSS version 23.0 statistical package (IBM Corp., NY, United States). Normal distribution within samples was assessed using the Kolmogorov-Smirnov test for normal distribution. Normally distributed data were presented as the mean ± SD. CBF value-derived 3D-ASL, target crossing times, distant in zone (%) platform, and total distance traveled were analyzed using the unpaired-sample Student *t* test between the normal and T2DM groups. Correlation coefficients between CBF and VEGF expression were calculated with the Spearman rank test. Multivariate analysis of variance was conducted for the time of escape latency for each group of rats. For behavior data, Sidak's multiple comparisons test was conducted to correct multiple comparisons using a two-way ANOVA. For behavioral parameters and to compare VEGF levels, statistical analyses were carried out using GraphPad Prism 8.0 (GraphPad Software, Inc., CA, United States). In addition, a P value < 0.05 was considered significant.

RESULTS

Rats with T2DM performed neurobehavioral abnormality

The MWM test was conducted to explore the learning performance and spatial memory ability of experimental rats. The positioning cruise experiments showed that

the swimming trajectory of the rats in the T2DM group was more chaotic (Figure 1). It was reflected as a significant increase in the time of escape latency compared with the normal group (F = 21.07, P < 0.0001) (Table 1). The total distance traveled was longer in the T2DM group than in the control group (t = 2.053, P = 0.003), and the platform was crossed fewer times (t = 2.491, P = 0.006). The percentage of distance in the zone target significantly decreased (t = 1.447, P = 0.020) (Figure 2). In the present study, greater time spent in the T2DM group was also reflected in the probe testing on day 5 of the experiment. These data indicated that the spatial learning memory ability in the T2DM group significantly decreased (Table 2).

Obvious reduction in CBF of rats with T2DM in the bilateral hippocampal area

CBF decreased in the bilateral hippocampal area in rats in the T2DM group. The left CBF value in the control and T2DM groups was 33.58 ± 2.91 mL/100 g/min and 27.20 ± 0.87 mL/100 g/min (t = 2.772, P = 0.0093), respectively. The right CBF value in the control and T2DM groups was 38.62 ± 3.76 mL/100 g/min and 29.0 ± 0.98 mL/100g/min (t = 3.373, P = 0.0020), respectively (Figure 3). No difference was observed between the right CBF value and the left CBF value in the T2DM group (P = 0.173).

Decreased CBF in rats with T2DM might be due to VEGF expression in the hippocampus

The expression of VEGF was lower in the T2DM group than in the control group (t = 2.768, P = 0.0325) (Figures 4 and 5). VEGF expression positively correlated with the left CBF value (*rho* = 0.776, P < 0.01) and the right CBF value (*rho* = 0.790, P < 0.01) (Figure 6). These data suggested that CBF in rats with T2DM might develop, at least partially, due to the decreased expression of VEGF.

CBF is an imaging biomarker of DACD

The escape latency negatively correlated with the CBF value (*rho* = -0.909, P < 0.01). The number of rats crossing the platform positively correlated with the CBF value (*rho* = 0.702, P < 0.05). A significant positive correlation was found between CBF and distance in the zone target (*rho* = 0.587, P < 0.05) (Figure 7).

DISCUSSION

Behavioral methods, including the MWM test, were used in this study to detect the changes in cognitive function. The changes in CBF in a rat model of T2DM were observed compared with those in the control group. In addition, the relationship between VEGF expression, CBF, and DACD was further explored. Several studies confirmed that T2DM led to cognitive impairment[10,30], but the mechanisms were unclear. The present study showed that the spatial memory and the reference memory in the MWM test were impaired in the T2DM group compared with the control group. CBF in the hippocampus of rats in the T2DM group was significantly reduced and was positively correlated with the data obtained from MWM, suggesting that low perfusion in the hippocampus was indeed associated with DACD. The correlation analysis also supported the conclusion that hypoperfusion in the hippocampus was indeed a risk factor for DACD[31,32]. However, no difference was found between the right CBF value and the left CBF value in the T2DM group. The study confirmed that decreased CBF in the hippocampus could be considered as an imaging biomarker to predict the risk of DACD, which was consistent with previous findings[24,33].

The hippocampus is an important anatomical structure related to cognition, particularly memory function. The hippocampal region is prone to suffer from cerebral microvascular disease due to its thin blood vessels and the relative lack of capillary anastomoses[15,34]. Therefore, it was speculated that the pathological changes in small vessels caused by a hyperglycemic environment could easily lead to a reduction of hippocampal CBF. Decreases in regional blood flow in the hippocampus can contribute to an ischemic and hypoxic environment, leading to neuronal damage in the hippocampus and cognitive impairment[35,36]. Previous studies focused mainly on the effect of hypoperfusion on cognition. No experimental study explored how VEGF expression in the hippocampus affected CBF based on 3D-ASL under the influence of long-term hyperglycemia in a T2DM rat model. In this study, the effect of VEGF on hippocampal perfusion in diabetes was studied by immunofluorescence detection of VEGF. The results showed that the expression of VEGF in the hippocampus was

Table 1 Results of the Morris water maze escape latency time in rats

Time interval	Control group, s	T2DM group, s	SD	*P* value
Day 1	58.32	53.66	4.655	0.9543
Day 2	34.45	44.44	-9.987	0.4580
Day 3	20.91	40.82	-19.91	0.0104[a]
Day 4	15.45	34.53	-19.07	0.0155[a]
Day 5	12.58	29.98	-17.41	0.0334[a]

[a]$P < 0.05$. SD: Standard deviation; T2DM: Type 2 diabetes mellitus.

Table 2 Results of the Morris water maze test in rats

Behavior parameter	Control group	T2DM group	Test value, *t*	*P* value
Target crossing times	2.50 ± 0.29	1.05 ± 0.21	2.491	0.006[b]
Distant in zone platform, %	0.95 ± 0.12	0.54 ± 0.10	1.447	0.020[a]
Total distance traveled in cm	137.30 ± 17.87	227.30 ± 16.89	2.053	0.003[b]

[a]$P < 0.05$.
[b]$P < 0.01$. T2DM: Type 2 diabetes mellitus.

significantly lower in the T2DM group compared with the control group, and positively correlated with CBF. Nevertheless, previous experimental findings on the changes in VEGF expression in the hippocampus in diabetes were controversial[37-39].

Previous data indicated that different VEGF levels might be due to the different stages of diabetes. High VEGF expression mainly existed in the early stage of diabetes, while low levels were seen in the late stage[40]. In the present study, the T2DM rat model (15 wk after STZ injection) already developed diabetic complications, which usually occurred in the late stage[41]. Thus, it was speculated that the low level of VEGF expression might be related to long-term hyperglycemia and late stage of the disease. However, this needs further investigation. A positive correlation was observed between decreased VEGF signaling and low CBF in the present study. Abnormal VEGF signaling caused by diabetes can lead to vascular dysfunction and pathological vessel remodeling, leading to vascular occlusion and insufficient blood supply[42-45]. Meanwhile, some studies found that the inhibition of VEGF signaling affected hippocampal dentate gyrus microvasculature and caused impairment in spatial memory[46]. Therefore, the decreased expression of VEGF in the late stage of diabetes led to hypoperfusion in the hippocampus, as found in the present study, leading to cognitive abnormalities.

This study was novel in investigating the correlation between CBF based on 3D-ASL, VEGF expression, and cognition in a T2DM rat model. However, it had certain limitations: (1) Previous studies revealed that the effect of VEGF expression on memory was primarily a result of axonal loss, demyelination, or changing plasticity of mature neurons[12,39,47]. The possibility that the decreased VEGF level might cause DACD *via* hippocampal neural injury was not examined in the present study; and (2) Previous findings indicated that each division of the hippocampus had different functions[48]. In the present study, CBF and VEGF expression in each hippocampal region were not examined. Thus, future studies are needed for detailed investigation.

CONCLUSION

Low perfusion of the hippocampus was associated with DACD. VEGF expression decreased in the hippocampal area of rats in the T2DM group in long-term hyperglycemia. Positive correlations were observed between CBF and VEGF expression in the hippocampus of rats with T2DM. Decreased CBF and low VEGF levels in the hippocampus might be risk factors of DACD. CBF measured by 3D-ASL might serve as a noninvasive imaging biomarker for detecting cognitive impairment

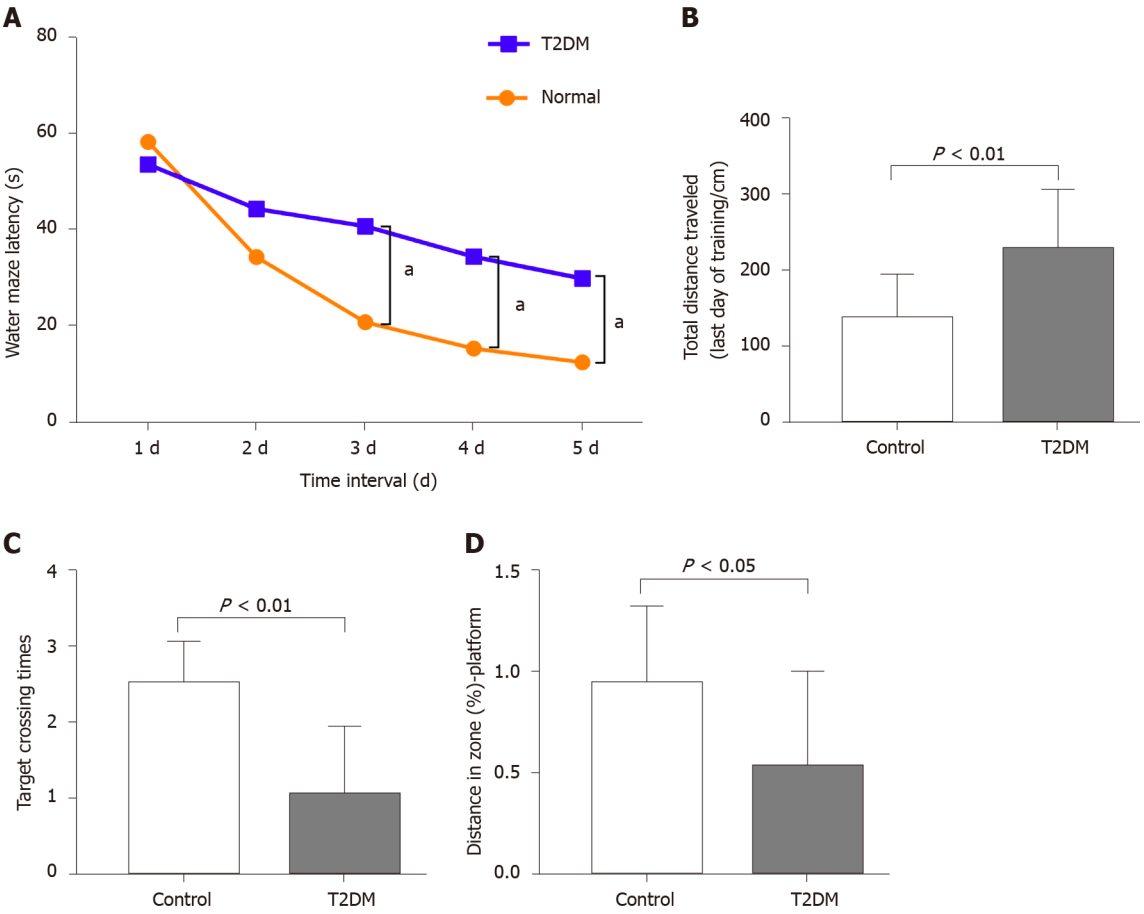

Figure 2 Results of the Morris water maze test of each group. A: Latency time on the third, fourth, and fifth day ($^{a}P < 0.05$); B: The total distance reach the platform was recorded in the hidden platform tests on the fifth day ($P < 0.01$); C: Number of crossing times of the target platform within 2 min ($P < 0.01$); D: Effects of different groups on the distance in zone-platform ($P < 0.05$). T2DM: Type 2 diabetes mellitus.

associated with T2DM.

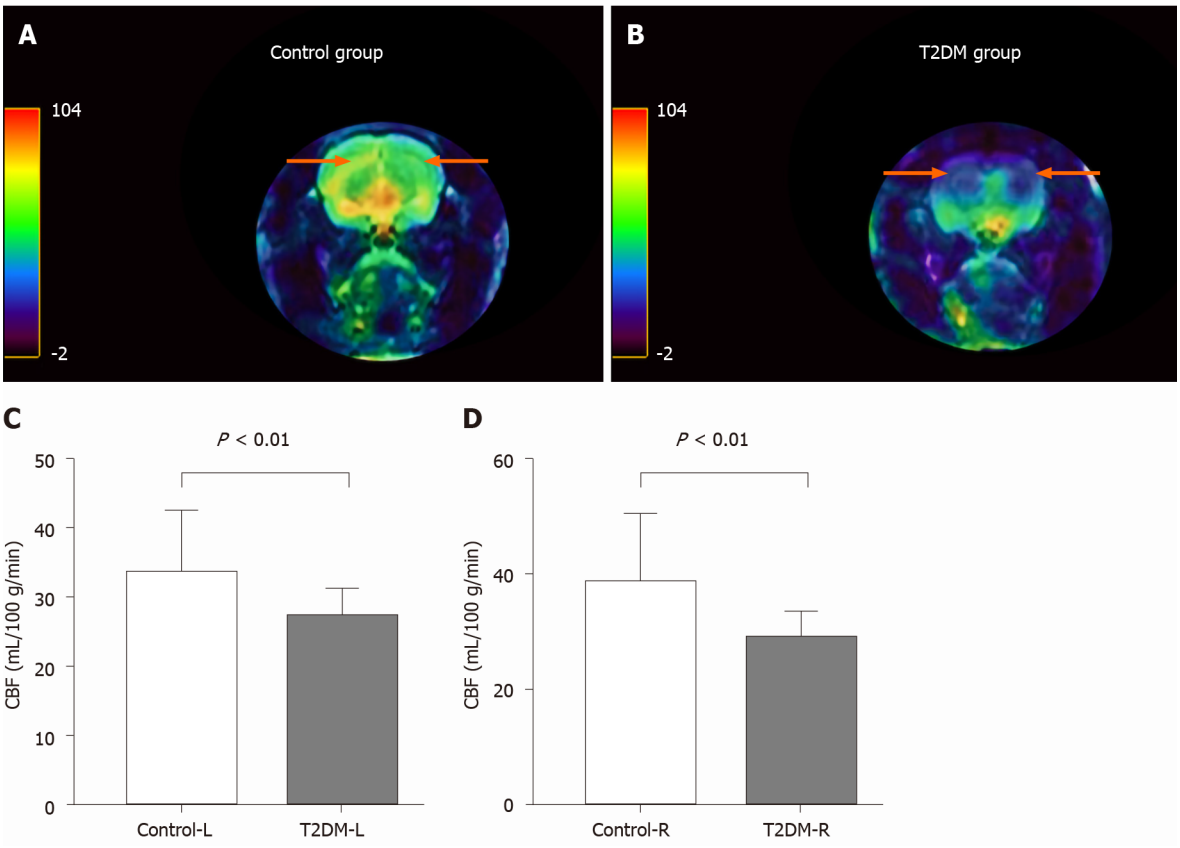

Figure 3 Comparison of cerebral blood flow in hippocampal area between type 2 diabetes mellitus group and control group. A: Representative cerebral blood flow (CBF) images of the bilateral hippocampus area (orange arrow) in the control group; B: Representative CBF images of the bilateral hippocampus area (orange arrow) in type 2 diabetes mellitus (T2DM) group; C: Significantly different CBF values between the control and T2DM groups in the left hippocampus ($P < 0.01$); D: Significantly different CBF between the control and T2DM groups in the right hippocampus ($P < 0.01$).

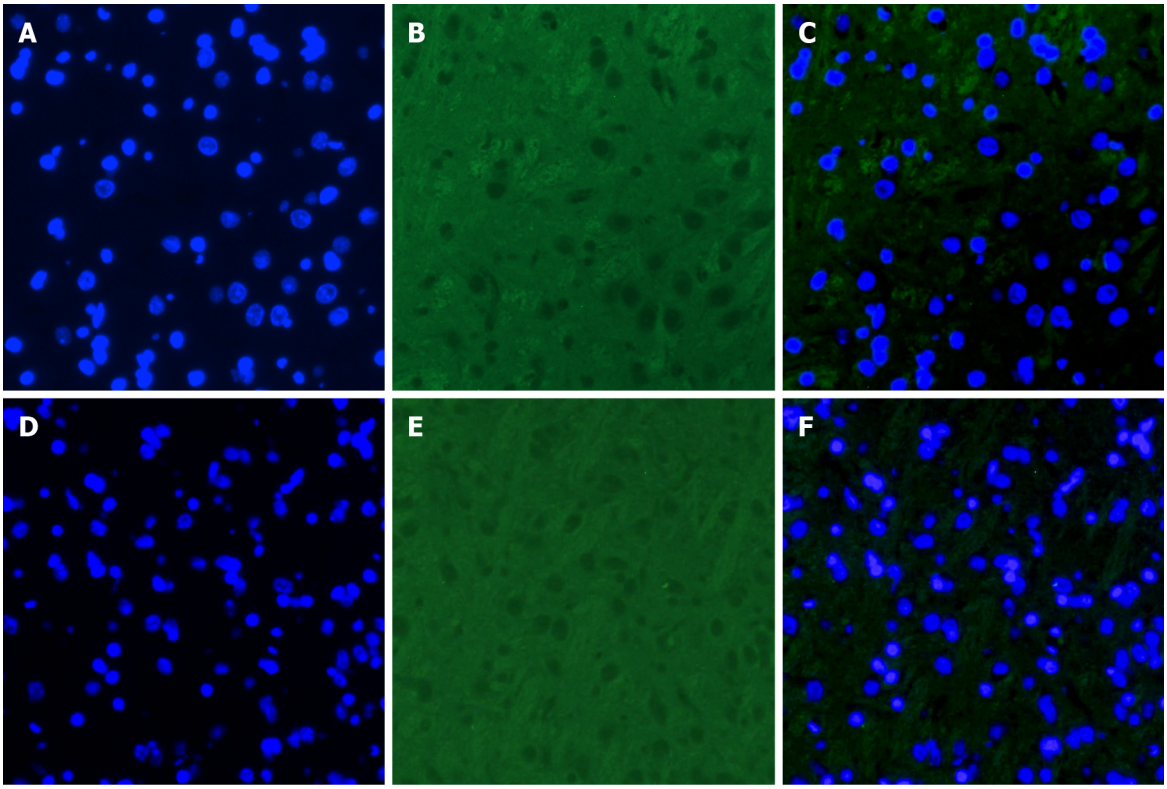

Figure 4 Images of vascular endothelial growth factor immunofluorescence staining of the hippocampus in each group. A: Nucleus in the type 2 diabetes mellitus group (blue); B: Vascular endothelial growth factor levels in the type 2 diabetes mellitus group (green); C: Merged image for the type 2 diabetes mellitus group; D: Nucleus in the control group (blue); E: Vascular endothelial growth factor levels in the control group (green); F: Merged image for the control group. Scale bar, 100 μm.

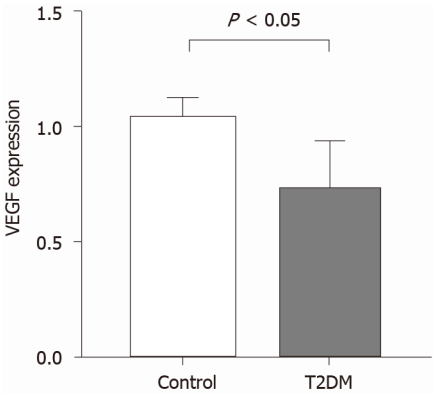

Figure 5 Expression of vascular endothelial growth factor was lower in the type 2 diabetes mellitus group than in the control group (*P* < 0.05). T2DM: Type 2 diabetes mellitus; VEGF: Vascular endothelial growth factor.

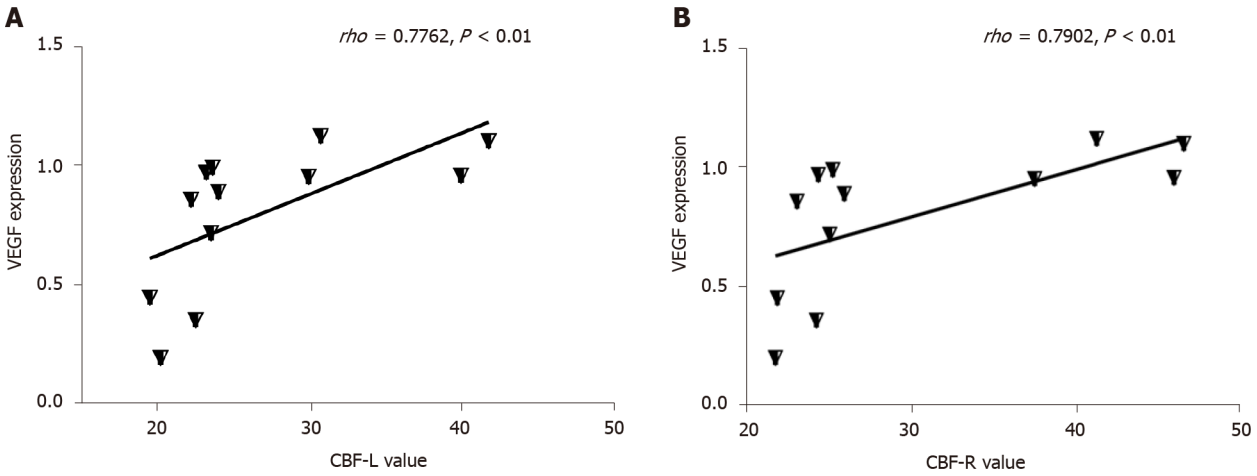

Figure 6 Good relationship was found between cerebral blood flow value and vascular endothelial growth factor expression among the type 2 diabetes mellitus group. A: Positive correlation between left cerebral blood flow (CBF) and vascular endothelial growth factor (VEGF) (*rho* = 0.7762, *P* < 0.01); B: Positive correlation between right CBF and VEGF (*rho* = 0.7902, *P* < 0.01). Unit for CBF value is mL/min/100 g. T2DM: Type 2 diabetes mellitus.

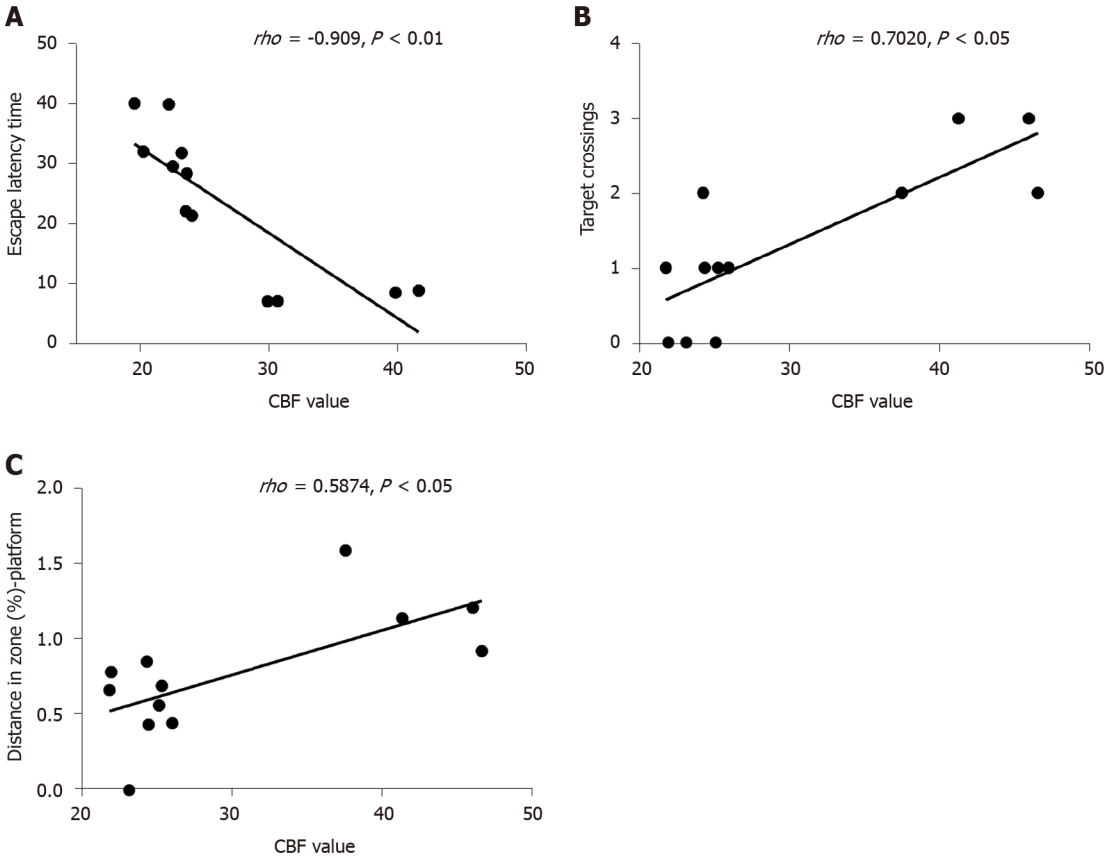

Figure 7 Correlation between cerebral blood flow value and cognitive dysfunction. A: The escape latency negatively correlated with the cerebral blood flow (CBF) value (*P* < 0.01); B: The number of rats crossing the platform positively correlated with the CBF value (*P* < 0.05); C: A significant positive correlation was found between CBF and distance in the zone target (*rho* = 0.587, *P* < 0.05).

ARTICLE HIGHLIGHTS

Research background

The mechanisms of diabetes-associated cognitive dysfunction (DACD) have not been fully elucidated to date. Some studies proved that lower cerebral blood flow (CBF) in the hippocampus was associated with poor executive function and memory in type 2 diabetes mellitus (T2DM). Increasing evidence showed that diabetes leads to abnormal vascular endothelial growth factor (VEGF) expression and CBF changes in humans

and animal models. This study explored whether DACD was correlated with CBF alteration and VEGF expression in the hippocampus.

Research motivation

Our study aimed to assess the relationship among CBF alteration, VEGF expression in the hippocampus, and DACD. Our findings may help reveal the mechanisms of DACD. This study would help in the detection of DACD and guide treatment.

Research objectives

This study aimed to explore whether VEGF signaling in the hippocampus in the T2DM rat model was related to CBF (measured by three dimensional arterial spin labeling) and DACD.

Research methods

Forty specific-pathogen-free grade Sprague-Dawley male rats were randomly divided into normal control and T2DM groups. The T2DM group was kept on a high-fat diet and then streptozotocin was administered by intraperitoneal injections to induce diabetes. The Morris water maze test was conducted to explore the learning performance and spatial memory ability of experimental rats. CBF measured by three dimensional arterial spin labeling was detected in the bilateral hippocampus. Immunofluorescence of VEGF in the bilateral hippocampus was performed, and VEGF expression was quantified with quantitative real-time PCR.

Research results

Our data indicated that the spatial learning memory ability in the T2DM group significantly decreased. An obvious reduction in CBF in rats with T2DM in the bilateral hippocampal area was observed. The expression of VEGF was lower in the T2DM group than in the control group. VEGF expression positively correlated with the CBF value in the hippocampus. A significant correlation was found between CBF and the spatial learning memory ability in the T2DM group.

Research conclusions

The new theories of this study was low perfusion of the hippocampus was associated with DACD and decreased VEGF expression in the hippocampal area of rats in the T2DM group in long-term hyperglycemia. To the best of our knowledge, this was the first study to explore the relationship of DACD, VEGF expression, and CBF of the hippocampus.

Research perspectives

Decreased CBF and low VEGF levels in the hippocampus might be risk factors for DACD. CBF measured by three dimensional arterial spin labeling might serve as a noninvasive imaging biomarker for detecting cognitive impairment associated with T2DM.

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