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REVIEW

Markers of insulin resistance in Polycystic ovary syndrome women: An update

Chantal Anifa Amisi

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Abstract

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, affecting 5%-10% of women of reproductive age. The importance of this syndrome lies in the magnitude of associated comorbidities: infertility, metabolic dysfunction, cardiovascular disease (CVD), plus psychological and oncological complications. Insulin resistance (IR) is a prominent feature of PCOS with a prevalence of 35%-80%. Without adequate management, IR with compensatory hyperinsulinemia contributes directly to reproductive dysfunction in women with PCOS. Furthermore, epidemiological data shows compelling evidence that PCOS is associated with an increased risk of impaired glucose tolerance, gestational diabetes mellitus and type 2 diabetes. In addition, metabolic dysfunction leads to a risk for CVD that increases with aging in women with PCOS. Indeed, the severity of IR in women with PCOS is associated with the amount of abdominal obesity, even in lean women with PCOS. Given these drastic implications, it is important to diagnose and treat insulin resistance as early as possible. Many markers have been proposed. However, quantitative assessment of IR in clinical practice remains a major challenge. The gold standard method for assessing insulin sensitivity is the hyperinsulinemic euglycemic glucose clamp. However, it is not used routinely because of the complexity of its procedure. Consequently, there has been an urgent need for surrogate markers of IR that are more applicable in large population-based epidemiological investigations. Despite this, many of them are either difficult to apply in routine clinical practice or useless for women with PCOS. Considering this difficulty, there is still a need for an accurate marker for easy, early detection and assessment of IR in women with PCOS. This review highlights markers of IR already used in women with PCOS, including new markers recently reported in literature, and it establishes a new classification for these markers.

Key Words: Markers; Insulin resistance; Polycystic ovary syndrome; Emerging markers; Impaired glucose tolerance

Core Tip: Diagnosing insulin resistance in Polycystic ovary syndrome is of crucial importance for better management and prevention of complications. Seeking of an easy-to-detect surrogate marker of insulin resistance represents a promising approach for maximizing treatment outcomes. This review highlights markers of insulin resistance already used in women with Polycystic ovary syndrome, including new markers recently reported in literature, and establishes a new classification of them.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder, affecting 5%-10% of women of reproductive age.

According to the Rotterdam consensus[1], it is defined by at least two of the following abnormalities: oligo- and/or anovulation, clinical and/or biological hyperandrogenism, and polycystic ovaries.

The importance of this syndrome lies in the magnitude of associated complications[2,3]: Reproductive complications: menstrual dysfunction, infertility, hyperandrogenism, increased pregnancy complications, amongst others; Metabolic complications: insulin resistance and increased risk factors for type 2 diabetes (T2D) mellitus and cardiovascular disease (CVD); Oncological complications: Endometrial, ovarian and breast cancers; Psychological complications: Heightened anxiety, depression.

Insulin resistance (IR), the most common metabolic feature, is found in almost 35%-80% of PCOS women and is independent of body mass index (BMI) and body fat distribution[4-7].

IR is usually defined as a pathological condition characterized by a decreased responsiveness or sensitivity to the metabolic actions of insulin. It is an established predictor of a range of disorders. In women with PCOS, IR plays an important role in the development and persistence of this disorder[8,9] and is recognized to lead to many of the metabolic abnormalities associated with metabolic syndrome. PCOS patients with IR are likely to have chronic subclinical inflammation and impaired fasting plasma glucose levels, which in turn enhance the prevalence of the more atherogenic, low-density cholesterol (LDL-c) particles[10].

Given this high prevalence, the need for accurate screening of IR in women with PCOS is obvious.

Early recognition and management of IR in women with PCOS would offer important preventive measures[11].

MARKERS OF DIRECT MEASUREMENT OF INSULIN RESISTANCE IN PCOS WOMEN

Hyperinsulinemic euglycemic clamp

The hyperinsulinemic euglycemic clamp technique is the gold standard method for assessing beta-cell sensitivity in humans, quantifying the amount of glucose metabolized by the body following a controlled hyperglycemic stimulus[12]. It has been used in cross-sectional and prospective studies designed to test insulin sensitivity in women with PCOS[9,13-17] and the effect of interventions such as pharmacological treatment and lifestyle management (weight loss, weight gain, or diet changes)[18-26].

However, the glucose clamp is irrelevant for clinical practice. It is ill-suited for large-scale investigations because of extensive requirements in procedure, cost, time and technical expertise. Therefore, it is rarely used.

SURROGATE MARKERS OF INSULIN RESISTANCE IN WOMEN WITH PCOS

Since the glucose clamp is difficult to apply in large-scale investigations because of the chaotic procedure, surrogate markers are obviously needed. Over the years, simple markers have been developed and used in clinical practice. They include anthropometric and biological indices.

ANTHROPOMETRIC MARKERS

Anthropometry has been widely and successfully used for assessing health and nutritional risk. Several hundred papers have been published in the past five decades that have reported the close relation between different measures of body size and one or another cardiovascular risk factors[27-34]. Most of them have attempted to assess the robustness and nature of these associations. Thus, several measures have been described and proposed as surrogate markers of IR. Anthropometric markers could be divided into fat anthropometric markers and bone anthropometric markers. To date, bone anthropometric markers have been reported as the best anthropometric marker for insulin resistance.

Fat anthropometric markers

BMI: BMI is the ratio of weight to the square of height, initially described by Keys in 1976[35]. BMI has traditionally been the chosen method to measure body size in epidemiological studies. It is used as a measure of overall adiposity and a good marker of variability in energy reserves in individuals with a sedentary lifestyle[35-41]. The positive association between obesity and the risk of developing T2D has been repeatedly observed, both in cross□sectional studies and in prospective studies[36-40].

Over the years, BMI has been shown to be an accurate marker for detecting cardiovascular risk. BMI > 25 kg/m² is a major risk factor for a wide range of chronic diseases and metabolic abnormalities, including T2D and IR[35-40].

In women with PCOS, BMI is an independent predictor of IR[41-44]; however, it is not routinely used as a surrogate marker of IR. Indeed, since IR in PCOS is independent of body fat, it could not accurately be predicted by BMI in lean PCOS women. BMI correlates more closely with IR in overweight and obese women than in lean PCOS women.

Waist circumference: First suggested by Lean *et al*[45], to be more strongly associated with metabolic risk than BMI, the stronger positive association between cardiovascular risk factors and abdominal adiposity measured by anthropometric measurements of abdominal circumference has been confirmed by several studies[45].

According to the World Health Organization (WHO), waist circumference (WC) is measured at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest [46].

WC is an easy surrogate marker of visceral adiposity and is commonly used in daily medical practice to detect IR clinically. Increased visceral adiposity is associated with a range of metabolic abnormalities, including decreased glucose tolerance, reduced insulin sensitivity and adverse lipid profiles, which are risk factors for T2D and CVD. Moreover, WC is the core component of the definition of metabolic syndrome. It is specifically required for diagnosing metabolic syndrome according to the International Diabetes Federation and the 2003 Rotterdam consensus[1,47].

A considerable correlation has been found between WC and insulin resistance assessed by the hyperinsulinemic euglycemic clamp technique[48]. A wide WC > 80 cm has been shown to be associated with IR in women with PCOS[49]. Therefore, WC is now considered the most clinically relevant approach for the measurement of IR[50].

However, the use of WC, a fat anthropometric marker, for the assessment of IR in women with PCOS is limited because IR is independent of visceral adiposity[5,7,44]. Several studies have failed to show an association between WC and IR in lean women with PCOS [51]. WC could predict IR in overweight and obese PCOS women but not in lean PCOS women[5,51]. Consequently, it is not a good anthropometric surrogate marker for assessing IR in women with PCOS[44].

Waist-to-hip ratio: The waist-to-hip ratio (WHR) is an anthropometric index that combines waist and hip measurements. It is used as a measure of body fat distribution. According to the WHO, WHR is calculated as waist circumference divided by hip circumference[46]. WHR > 0.8 corresponded with a BMI overweight range of 25-29.9 kg/m².

Since it measures abdominal obesity, which in turn is attributed to the presence of visceral adipose tissue that promotes insulin resistance, WHR is used as a predictor of IR and metabolic risk. However, it has been described in several papers as a less accurate marker of adiposity that could predict cardiovascular and metabolic risk[27,44,52].

In PCOS assessment, its used has been practically abandoned[27,44,52].

Waist-to-height ratio: In the middle of the 1990s, the use of waist-to-height ratio (WHtR) was first proposed by Lee *et al*[32], for detecting abdominal obesity and associated health risks[53].

WHtR is calculated as waist divided by height.

Several studies have found a strong association of WHtR with cardiovascular risks. Indeed, it has been reported as the best anthropometric marker to assess T2D, metabolic syndrome, cardiovascular events, and altered blood pressure[53-57]. According to Ashwell *et al*[54], WHtR is one of the best alternative measures in predicting chronic diseases. In a systematic review comparing WC to WHtR, they found that the use of WHtR provided better results over WC for CVD outcomes, as well as for T2D and hypertension. In addition, Huxley *et al*[27] conducted a systematic review and meta-analysis of the anthropometric indices of cardiometabolic risk factors, involving 32 studies, to determine which of the

four indices (BMI, WC, WHR and WHtR) is the best discriminator of major cardiovascular risk factors. They found that measures of central obesity were superior to BMI as discriminators of risk of T2D, and therefore of IR. Huang *et al*[58], concluded that WHtR is one of the most representative marker to assess insulin resistance. The superiority of WHtR over BMI for detecting cardiovascular risk factors has been reported in a meta-analysis[59].

In women with PCOS, a few articles using WHtR as a marker are available. In a study conducted by Costa *et al*[60], in Brazilian women with PCOS, WHtR was the marker that presented significant positive correlations with the highest number of cardiovascular risk factors. They proposed the inclusion of this easily-measured parameter in the clinical assessment for the screening of women with PCOS and cardiovascular risk factors. Similarly, the results of a study by Gateva *et al*[61], indicated that both WHtR and WC, but not WHR, were good markers of adverse metabolic profiles in women with PCOS. More recently, Bhattacharya *et al*[62], suggested that WHtR could be used as an inexpensive and noninvasive screening tool for the early prediction of PCOS and IR among PCOS patients. Amisi *et al*[44], comparing several anthropometric markers, found that WHtR and WC showed similar performance but were less predictive of IR than wrist circumference.

Bone anthropometric markers

Wrist circumference: Wrist circumference (Wrc) was first proposed as a marker of insulin resistance in young obese people by Cappizzi *et al*[63]. His team was inspired by the findings of Karsenty *et al*[64], on the involvement of the bone system in glucose metabolism *via* osteocalcin (OC) effects on insulin[65-67]. Hyperinsulinemia has been associated with increased bone mass[68-70], and wide WrC has been associated with IR[71-74]. Esmaeilzadeh *et al*[75], found a positive correlation between WrC and PCOS status.

Amisi *et al*[44], showed that WrC is the best anthropometric marker known to date for the assessment of insulin resistance in women with PCOS. In their study, they reported a significantly higher correlation of nondominant WrC with IR than other anthropometric markers.

The novelty of WrC as a marker of IR is that it is based on the assessment of IR on bone, not on fat, as other anthropometric markers.

WrC is, to date, the only anthropometric marker that can assess IR in both obese and lean women. WrC is, consequently, the only useful clinical measure for assessing IR in lean women with PCOS. Given that most women with PCOS are insulin resistant, which is independent from fat and characterized by hyperinsulinemia, fat anthropometric markers are not suitable[44].

However, there are few publications on WrC as a marker of IR in women with PCOS.

BIOLOGICAL MARKERS

Markers using insulin and/or glucose

Oral glucose tolerance test: The oral glucose tolerance test (OGTT) is a frequently used index of glucose tolerance. It is commonly used in medical practice to detect IGT and T2D. Moreover, OGTT is the only means of identifying people with IGT[76]. The WHO recommends the test as a valid way to diagnose diabetes.

According to the WHO, the OGTT technique involves the oral administration of 75 g of glucose after 8 to 10 h of fasting. At 0, 30, 60 and 120 min following the oral glucose load, blood glucose levels are measured to determine how rapidly it is cleared from the bloodstream.

In PCOS women, the Androgen Excess Society in consensus with the European Society of Human Reproduction and Embryology (ESHRE) and the American Society of Reproductive Medicine (ASRM) have recently recommended a 2 h OGTT in all women with PCOS, with annual or biannual rescreening, depending on the risk factors[11,77,78]. The Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS consensus Workshop Group recommended screening for IGT and T2D when presented with the following conditions: hyperandrogenism with anovulation, acanthosis nigricans, obesity (BMI > 30 kg/m^2) in women with a family history of T2D or gestational diabetes mellitus[78].

However, OGTT provides useful information about glucose tolerance but not insulin resistance. In addition, it is more time-consuming and labor intensive to perform.

Glucose/insulin ratio: The glucose/insulin ratio (G/I) has long been employed as an index of IR[4,79-82].

It has been described by Legro *et al*[83], as a useful measure of insulin sensitivity in obese PCOS women and has both high sensitivity and specificity for detecting IR in women. In addition, the G/I ratio reflects profound peripheral IR and hepatic IR, which are found in obese women.

Furthermore, Quon confirmed the same in his editorial published in 2004, explaining how the G/I ratio correlates with insulin sensitivity in nondiabetic patients with PCOS[84]. In healthy subjects with normal fasting glucose levels, elevations in fasting insulin levels correspond to increased IR. Since fasting glucose levels are similar for all subjects, the G/I ratio is functionally equivalent to 1/insulin, which is a well-known proxy for insulin sensitivity. It decreases as a subject becomes more insulin

resistant and their fasting glucose rises[84].

However, the use of the fasting G/I ratio is limited in PCOS women with abnormal fasting glucose levels because, as demonstrated by Quon, this leads to erroneous results. Indeed, the G/I ratio is similar to 1/insulin in nondiabetic subjects, but it increases paradoxically in diabetic subjects and in PCOS women with abnormal glucose levels[84]. Consequently, the fasting G/I ratio has been considered a potentially flawed index of insulin sensitivity[84].

Fasting insulin: Numerous studies have investigated and proposed fasting insulin concentrations as the simplest index for assessing IR[85-87] because it has been shown to correlate well. High fasting insulin level in individuals with normal glucose tolerance has been found to reflect IR. Furthermore, high insulin concentrations presage the development of diabetes in the future[88].

In nondiabetic subjects with normal fasting glucose levels, the rise of fasting insulin levels corresponds to insulin resistance. In this population, insulin sensitivity, which decreases as subjects become more insulin resistant, can be substituted by 1/fasting insulin.

In women with PCOS, many authors have recommended fasting insulin as a simple office-based method to assess insulin resistance[77,89,90].

Recently, after comparing the prevalence of IR using published methods in a cohort of women with PCOS, Lunger *et al*[91], suggested the use of fasting insulin as a simple screening test. This can reduce the number of OGTTs needed to routinely assess IR in women with PCOS, as proposed by the Androgen Excess Society.

However, the use of fasting insulin for assessing IR in women with PCOS could be limited by a lack of adequate laboratories and the cost of insulin assays, especially in developing countries.

Minimal model analysis of frequently sampled intravenous glucose tolerance test: The frequently sampled intravenous glucose tolerance test (FSIVGTT) is an alternative method sought to simplify the clamp procedure. It provides information on both insulin sensitivity and β-cell function. The minimal model was developed by Bergman *et al*[92], in 1979 as a method to obtain an indirect measurement of insulin sensitivity or insulin resistance.

The standard technique of FSIVGTT includes multiple blood sampling for insulin and glucose. Baseline blood samples for insulin and glucose were taken at 15, 20, 25, and 30 min following the placement of an intravenous catheter. Glucose was then infused intravenously as a bolus over 1 min, followed by the extraction of blood samples for glucose and insulin measurements at 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160 and 180 min after the start of the glucose injection.

Plasma glucose and insulin concentrations collected during the test were subjected to minimal model analysis using the computer program MINMOD to generate an index of insulin sensitivity (Si).

Parameters derived from minimal model analysis have been found to correlate with those from euglycemic clamps[93].

Although FSIVGTT is minimally invasive and easier than euglycemic clamp, it is not suitable for large epidemiological studies. The complexity of the sampling procedure, the number of samples required and especially the corresponding higher cost make it unsuitable for clinical use.

Homeostasis model assessment: The Homeostasis Model Assessment (HOMA) is a method used to quantify insulin resistance from basal glucose and insulin levels, first described in 1985 by Matthews *et al*[94]. HOMA is a mathematical model of the relationship of insulin and glucose concentrations for a wide range of possible combinations of insulin resistance and β-cell function. It assumes the principle of interactions between β-cell deficiency, insulin resistance and fasting hyperglycemia. Consequently, any given decrease in insulin sensitivity and β-cell dysfunction is associated with fasting steady-state insulin and glucose concentrations. Using a computer-solved mathematical model of basal insulin and glucose interactions, the authors plotted a wide range of basal plasma insulin and glucose concentrations expected for possible combinations of insulin resistance and β-cell deficiency, to obtain the first model of HOMA. The early model was later updated using nonlinear solutions[95]. The approximating equation for insulin resistance has been simplified, and insulin resistance values can be derived from basal insulin and glucose concentrations as follows: HOMA-IR = insulin (mU/L) × glucose (mmol/L)/22.5.

The β-cell function is calculated as: HOMA β-cell = 20 x insulin (mU/L)/[glucose (mmol/L) - 3.5].

A strong linear correlation of HOMA-IR has been found with the euglycemic-hyperinsulinemic clamp [96,97]. However, HOMA-IR is determined from fasting concentrations of glucose and insulin. It provides an estimation of hepatic insulin sensitivity and could, therefore, assume the important limitation of identifying hepatic and peripheral insulin sensitivity. However, this is not the case in reality.

In women with PCOS, HOMA-IR has been used in various studies of distinct populations to assess insulin resistance[7,44,98-101]. Furthermore, the HOMA has proven to be a robust clinical and epidemiological tool for assessing IR. Similarly, HOMA β-cell has been used as a marker of basal insulin secretion by pancreatic β-cells[98].

In Sub-Saharan African women and in developing countries in general, HOMA-IR has been successfully used[7,44]. However, although the HOMA index has proven to be an accurate means to assess insulin resistance, it is difficult to perform in developing and low-resource countries because of

the cost of insulin measurements, as well as the lack of adequate laboratories and equipment.

Log (HOMA-IR): To more accurately reflect the physiology, other modifications have been made to the Homeostasis Model Assessment for insulin resistance (HOMA-IR). Using a computer program, log transformed HOMA-IR [Ln(HOMA-IR)] was obtained[96,102] and it correlates well with the eugylcemic clamp method[96].

Comparing Log(HOMA-IR) and HOMA-IR with the Minimal model, Log(HOMA-IR) correlated more strongly than HOMA-IR in nondiabetic subjects[103]. Log(HOMA-IR) has been found to be more convenient than HOMA-IR for the assessment of IR in mild to moderate diabetes and glucose intolerance. Moreover, Log(HOMA-IR) is a better predictor of insulin sensitivity than HOMA-IR[103].

Similar to HOMA-IR, log(HOMA-IR) has been extensively used in large epidemiological studies and in clinical research[103,104].

However, log(HOMA-IR) has been used in few studies for assessing IR in women with PCOS[42,105,106].

Fasting insulin resistance index: The fasting insulin resistance index (FIRI) was proposed by Duncan *et al*[107], in 1989.

FIRI is calculated as: FIRI = (glucose x insulin)/25.

However, in women with PCOS, FIRI has not been extensively used, similar to HOMA-IR[108,109].

Quantitative insulin sensitivity check index: Quantitative insulin sensitivity check index (QUICK) is an index of insulin sensitivity that provides a consistent and precise index of insulin sensitivity with better positive predictive power[110-111]. It is calculated from basal glucose and insulin concentrations obtained from a single fasting blood specimen. QUICKI is similar to HOMA and is simply its variation, as it interprets the data by taking both logarithms and the reciprocal of the fasting glucose-insulin product. Consequently, it is more accurate than HOMA in calculations over a wide range of insulin sensitivities.

QUICKI = 1/[log insulin (μU/mL) + log glucose (mg/dL)]

This formula implies that the lower the QUICKI value, the lower the insulin sensitivity.

QUICKI has been strongly correlated with measurements made by the euyglycemic clamp technique, especially in obese and diabetic subjects[112]. However, its performance was less satisfactory in subjects with normal glucose tolerance. Therefore, the revised QUICKI, which incorporates the fasting plasma free fatty acid concentration (FFA) into the equation, has been proposed[113-114]:

Revised QUICKI = 1/[log insulin (μU/mL) + log glucose (mg/dL) + log FFA (mmol/L)]

QUICKI has been shown to be appropriate and effective for use in large epidemiological or clinical research studies[111,115].

In a large meta-analysis of insulin-resistant subjects, Hanley *et al*[115], demonstrated that QUICKI is a simple surrogate index with the best positive predictive power for determining the development of diabetes.

In women with PCOS, QUICKI is among the most thoroughly evaluated surrogate indices for insulin sensitivity. It has been validated as a simple, inexpensive, useful, and minimally invasive surrogate index of insulin sensitivity[116-118].

Derived surrogate markers from OGTT

Some studies, carried out in other clinical conditions, suggested that surrogate indices derived from the OGTT could perform better than those obtained from fasting values[119-122].

Matsuda index: Additionally, called "the composite index", the Matsuda index was described by Dr Masafuni Matsuda and Prof Ralph DeFronzo in 1999. The Matsuda index, or the composite whole-body insulin sensitivity index (WBISI), is an index of IR derived from the OGTT that evaluates whole-body physiological insulin sensitivity. It is determined by insulin and glucose values obtained from the OGTT [120].

In women with PCOS, Rizzo *et al*[123], found that the Matsuda index correlates well with the HOMA-IR and QUICKI, indicating that it may be a reliable substitute in the detection of IR and subsequent intervention required to improve outcomes in women with PCOS. Ciampelli *et al*[90], observed that the Matsuda index obtained the best correlation coefficients with the euglycemic clamp in menopausal women.

Stumvoll index: Another index derived from the OGTT has been described by Stumvoll *et al*[121]. From demographic data (age, BMI, WHR), as well as insulin and glucose values obtained from the OGTT, they found a new index to predict insulin sensitivity and beta cell function.

However, in PCOS, only a few published studies have used the Stumvoll index[121,124-126].

In a recent study, Lewandowski *et al*[124], found that the correlation between various IR indices is highly variable when comparing surrogate methods based on fasting insulin and either fasting glucose (HOMA-IR and QUICKI) or triglycerides (McAuley Index), with IR indices derived from glucose and insulin during an OGTT (Belfiore, Matsuda and Stumvoll indices). They suggested that the clinical application of surrogate indices for the assessment of IR in PCOS must therefore be viewed with extreme caution[124].

Tosi *et al*[119], evaluated the performance of several surrogate markers of insulin resistance in identifying individual PCOS subjects with impaired insulin sensitivity, as defined by the euglycemic clamp, and found that all surrogate indices were highly correlated with hyperinsulinemic euglycemic clamp values. However, their ability to identify insulin-resistant individuals was limited in terms of sensitivity, especially in normal-weight subjects. ROC analysis showed similar performances of these indices (AUC values 0.782-0.817). They concluded that surrogate indices of insulin action show a low sensitivity in identifying insulin-resistant subjects, which causes many subjects to be erroneously diagnosed as insulin sensitive[119].

Avignon index: Avignon *et al*[127], also used OGTT values to try and develop another insulin sensitivity index. They compared sensitivity indices obtained from baseline fasting insulin and glucose levels (Sib), and at the end of the second hour of the OGTT (Si2h), a third insulin sensitivity index (SiM) was calculated by averaging Sib and Si2h. They observed that sensitivity indices obtained were useful to obtain a single test that could be used to determine both glucose tolerance and an estimate of insulin sensitivity.

In the study conducted in women with PCOS and menopausal subjects, which aimed to verify the validity of several indices of insulin sensitivity by comparing the data obtained by indices to those of the euglycemic clamp, Ciampelli *et al*[90] found that the best correlation with clamp studies was obtained with the Avignon Insulin Sensitivity Index in PCOS. The Matsuda index obtained the best correlation in menopausal patients[90].

Gutt index: In the search for a simple measure of insulin sensitivity, Gutt *et al*[122], also explored the use of OGTT values.

They devised a formula for an insulin sensitivity index, ISI (0, 120), that uses the fasting (0 min) and 120 min post oral glucose (OGTT), insulin and glucose concentrations. They found that ISI (0, 120) correlates well when applied prospectively in comparative studies, with the insulin sensitivity index obtained from the euglycemic hyperinsulinelic clamp[122].

In PCOS, Tosi *et al*[119], demonstrated the substantial pitfalls of derived surrogate indices, including the Gutt index, in identifying insulin-resistant individuals among PCOS women. Collectively, these indices showed a high PPV (90%-96%) but a low NPV (36%-45%). In other words, many subjects with insulin resistance were not recognized by any of these surrogate markers[119].

Insulinogenic index: The insulinogenic index (IGI) is derived from the OGTT to evaluate β-cell function.

IGI = [(30 min insulin - fasting insulin)/30 min glucose]

IGI is used to estimate the level of insulin secretion during glucose administration. The insulinogenic index has been commonly used during the first 30 min of the OGTT as a surrogate measure of first-phase insulin responses to a glucose challenge[128].

In women with PCOS, IGI is frequently used to express β-cell function[9,129-132].

Homa-M120: Morciano *et al*[133], first reported the aim of developing and validating a specific simple measure of insulin sensitivity using oral glucose tolerance test (OGTT) values for lean PCOS women because their cardiometabolic impairment is more frequently misunderstood. They showed that a temporarily delayed assessment of glucose and insulin concentrations during OGTT is more predictive of IR than a standard fasting evaluation, such as with HOMA-IR[133].

They then compared HOMA-M120 with other OGTT-derived indices and concluded that the 120-minute glucose and insulin evaluation (HOMA-M120) was the best IR index in lean PCOS women[133].

Song DK *et al*[126], made the same observation that lean women with PCOS, even when β-cell function is matched, showed higher values for HOMA-M120 but not HOMA-IR than matched controls.

Markers using lipid and lipoproteins

Abnormal lipid metabolism is one of the main characteristics of women with PCOS, with a prevalence of up to 70%[134-136]. Insulin resistance is closely associated with lipid disorders: elevated triglycerides (TGs), low-density cholesterol (LDL-c) levels and low high-density cholesterol (HDL-c) levels[136-142]. Increased serum concentrations of LDL-c and TG, as well as decreased HDL-c, are recognized as risk factors for cardiovascular disease[143-145]. Several epidemiologic studies have reported that lipid ratios are better predictors of atherosclerosis and cardiovascular disease than any other single lipid marker [144]. The superior ability of lipid ratios to predict the risk of cardiovascular disease than single lipid markers is of particular clinical interest.

Seeking a simple, effective and economic method to investigate IR, many researchers have suggested lipid ratios as surrogate indices[138-142].

Moreover, in PCOS patients, several studies have shown that the serum lipoprotein ratio has a significant positive correlation with IR and could be employed as a simple reliable indicator to determine IR[134-142,146].

TG/HDL-c: In overweight individuals with normal glucose tolerance, the TG/HDL-c ratio has shown the ability to identify IR with similar sensitivity and specificity to those of fasting plasma insulin concen-

tration. It has been proposed as a marker of insulin resistance[147]. Furthermore, low serum HDL-c combined with increased serum TG concentrations predicts the development of T2D[148].

In women with PCOS, Barrios *et al*[149], evaluated the relationship between the TG/HDL-c ratio and IR indices. They found that women with PCOS showed significantly higher TG/HDL-c ratios and HOMA-IR values, but lower QUICKI values. They proposed the TG/HDL-c ratio as a useful and practical method of assessing IR[149]. The same observation was made by Xiang *et al*[139]. The TG/HDL-c ratio seems to be the best index that directly correlates with insulin levels and can therefore be used as a marker of IR[138-140,149].

However, the problem with all markers using TG levels is that they could not be used efficiently in the African population because of the presence of TGs. Indeed, the Sub-Saharan African population presents what has been called the "TG paradox": Normal TG levels in the presence of IR[150]. This fact emphasized the previous need for a normal threshold of TG in the African population.

TC/HDL-c: Several epidemiologic studies have demonstrated that total cholesterol (TC)/HDL-c is a better predictor of atherosclerosis and cardiovascular disease than TC or HDL-c alone[144]. Furthermore, the TC/HDL-c ratio was shown to correlate negatively with insulin concentrations[151]. Subsequently, normal subjects with standard weight or overweight, as well as an increased TC/HDL-c ratio, have shown insulin resistance, increased TG concentrations, and hyperinsulinemia[152].

In women with PCOS, upon comparing the three lipid ratios commonly used as surrogate markers of IR (TG/HDL-c, TC/HDL-c, LDL-c/HDL-c), Xiang *et al*[139], found that the area under the ROC curve of TC/HDL-c was the largest, with the highest sensitivity and specificity. However, these findings were not confirmed in a similar study that reported the largest area under the ROC curve of TG/HDL-c[140].

LDL/HDL-c: Another index using lipoprotein is LDL/HDL-c ratio. It has also been found to correlate well with cardiovascular diseases.

In women with PCOS, it has been shown that LDL/HDL-c is an effective diagnostic marker for insulin resistance[139-140].

Emerging markers

Scientific evidence has disclosed strong influences between inflammatory mechanisms and IR. Some studies have shown that insulin resistance itself amplifies chronic inflammation[153]. PCOS is now recognized as a proinflammatory state associated with elevations in a number of circulating inflammatory mediators[154]. Therefore, it is not surprising that inflammatory markers have gained popularity in IR assessment, with several being proposed as surrogate markers of IR.

Interleukin-6: Interleukin-6 (IL-6), a major proinflammatory cytokine, has been shown to be closely associated with IR[155].

In women with PCOS, low-grade chronic inflammation has been reported and is involved in the pathogenesis of T2D and CVD[156]. However, conflicting results regarding IL-6 Levels in women with PCOS have been reported.

To evaluate IL-6 Levels in women with PCOS, a systematic review and meta-analysis were performed [157]. High levels of IL-6 have been reported to be related to IR. Interestingly, IL-6 levels have been reported to be high in both lean and obese women with PCOS. Indeed, IL-6 has been found to be related to IR and androgen levels but not to BMI.

However, Escobar-Morreale did not find statistically significant differences between PCOS and controls regarding IL-6 concentrations[154].

C-Reactive protein: C-Reactive protein (CRP) is one of the markers of systemic subclinical inflammation [158,159]. The relationship of CRP and several measures of IR has been described[160]. However, CRP alone could not predict IR.

It is well known that women with PCOS exhibit an elevation in circulating CRP that is independent of obesity[161]. Moreover, in a meta-analysis, circulating CRP was found to be 95% higher in women with PCOS than in controls[154]. This finding corroborates the existence of low-grade chronic inflammation in women with PCOS[156,161].

Nonetheless, in women with PCOS, elevation of CRP seems to be a PCOS effect rather than a result of IR. This fact limits its use as a good marker of IR.

Soluble CD 36: Soluble CD36 (SCD36) was initially described by Handberg *et al*[161], as a novel marker of IR. It has been found to be distinctly elevated in patients with IR and T2D[161].

In PCOS, a study conducted by Glintborg *et al*[162], reported that SCD36 correlated with measures of insulin sensitivity independent of central fat mass. Furthermore, pioglitazone treatment reduced SCD36 while improving insulin-stimulated glucose metabolism[162].

Nonetheless, more studies need to be conducted in PCOS to ascertain this association.

C3 complement: Recently, Muscari *et al*[163], reported a strong link between C3 complement (C3) and IR in an elderly population, independent of the components of metabolic syndrome. Some researchers have described the insulin-like properties of C3. Indeed, activation of C3 complement has been proven

to have insulin-like properties. It affects glucose transmembrane transport and promotes the synthesis of TG in adipocytes[164].

In PCOS, Yang *et al*[165], reported a strong association of serum C3 complement with insulin resistance. Lewis RD *et al*[166], observed a similar phenomenon. However, in a study conducted by Dehdashtihaghighat *et al*[167], such an association was not found.

Even so, this observation needs to be further investigated.

Ferritin: Ferritin, a major intracellular iron storage protein, has been proposed as a new marker of IR. High levels of ferritin have been associated with hyperinsulinemia and hypertriglyciridemia[168].

In PCOS women, elevated serum ferritin levels have been found to be associated with increased insulin resistance and the risk of diabetes in obese women but not in nonobese women[169]. Moreover, in both obese and nonobese PCOS women, higher serum ferritin levels have been correlated with a greater risk of hypertriglyceridemia.

In addition, elevated ferritin levels have been reported as a result of insulin resistance and hyperinsulinism but not reduced menstrual losses secondary to oligomenorrhea or amenorrhea[170, 171].

Nevertheless, more studies are needed to better clarify its applicability as a marker of IR in women with PCOS.

Adiponectin: Adiponectin is a protein produced by adipocytes with direct insulin sensitizing activity, plus vascular protective and anti-inflammatory effects. Adiponectin reduces glucose production by the liver and increases fatty acid oxidation in skeletal muscle. In addition to its antidiabetic effects, adiponectin possesses direct antiatherogenic properties[172,173]. In a variety of conditions frequently associated with IR, such as diabetes, hypertension and CVD, its plasma concentration has been found to be reduced[174,175]. Moreover, a reduction in high molecular weight (HMW) adiponectin levels, a fraction of adiponectin that is considered a potent mediator of insulin sensitivity, has been reported in IR states[176]. HMW is also decreased by testosterone[177]. It has recently been proposed that the ratio of HMW/total adiponectin, but not the absolute amounts of adiponectin, determines insulin sensitivity [178].

In women with PCOS, low serum adiponectin and HMW levels have been reported to be associated with IR[8,179-181]. It has been suggested that adiponectin may serve as the common denominator that connects obesity, IR and altered lipid metabolism in PCOS patients[177]. Furthermore, serum adiponectin levels have been suppressed in patients with both metabolic syndrome and IR. Consequently, the use of serum concentrations of adiponectin as a biomarker for insulin resistance has been suggested to distinguish PCOS patients at a higher risk of diabetes and cardiovascular morbidity [182].

However, the assumption that adiponectin is an intrinsic characteristic of IR in women with PCOS remains controversial. In addition, the effect of testosterone levels on adiponectin levels should be further investigated.

Tumor necrosis factor-α: Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine produced mainly by monocytes and macrophages. Several studies have shown a relation between TNF-α and IR in the general population[183].

In women with PCOS, multiple studies have demonstrated elevated levels of TNF-α[184,185].

TNF-α has been shown to impact ovarian function, including follicular development, ovulation, and corpus luteum regression[186]. Furthermore, it has been suggested that TNF-α promotes IR in women with PCOS and is implicated in the pathophysiology of PCOS[185].

However, Escobar-Morreale *et al*[154], in a meta-analysis cited above, found that TNF-α levels were not significantly different in women with PCOS compared to controls.

Therefore, the association of TNF-α and IR in women with PCOS remains controversial.

Glycosylated hemoglobin: Glycosylated hemoglobin (HbA1c) is the most common marker of chronic hyperglycemia and has long been considered the most practical approach used to review long-term glycemic control in diabetic patients. However, in 2010, the American Diabetes Association (ADA) included a glycated hemoglobin A1(c) (A1C) level as a component of diagnostic criteria of 'increased risk for diabetes'[187]. Since then, some researchers have conducted studies to examine the relationship of 'elevated A1C' (≥ 5.7%) with 'increased risk for diabetes' in women with PCOS to generalize its use as a screening test of prediabetes[188-192]. Indeed, increased HbA1c levels in the range of 5.7%-6.4% have been found to reflect IR or some component of metabolic syndrome[193].

However, the results reported in the current literature are controversial. A high prevalence of elevated A1C in nonobese patients with PCOS and an increased risk of elevated A1C have been associated with PCOS. Therefore, assessment of A1C as a useful new approach to screening for diabetes has been recommended[188,194]. Conversely, many studies do not support the recommendation that HbA1c can be used for the screening of prediabetes in women with PCOS because it failed to identify IR, though it was diagnosed in many PCOS patients by HOMA or fasting insulin levels[190,195].

Leptin: Leptin is an adipocyte-derived hormone that regulates a broad spectrum of homeostatic functions. It was the first adipokine to be identified[195,196]. One homeostatic function modulated by leptin is the regulation of insulin secretion by pancreatic β-cells and the regulation of insulin action and energy metabolism in adipocytes and skeletal muscle[197]. Leptin suppresses food intake and promotes energy expenditure mainly *via* its direct effects on hypothalamic neurons, and it is thus considered an antiobese hormone. Leptin levels decrease with fasting and increase with food intake[198,199].

A positive relationship between leptin, fat mass and BMI has been reported. Leptin levels are increased in obesity and significantly correlated with IR[200].

In women with PCOS, several prospective studies have confirmed that an increased leptin level is associated with insulin resistance and an elevated risk of obesity and diabetes[201-203]. Leptin has been found to have a strong positive correlation with HOMA-IR[204]. However, many studies failed to report any significant differences in serum leptin levels in women with PCOS when compared with age- and weight-matched controls[205-207]. The relationship between leptin and IR is thus still a matter of debate. Wang *et al*[208], did not observe significant differences in serum leptin between PCOS with IR and PCOS without IR. However, Yildizhan *et al*[202], observed an association between serum leptin levels and IR in young women with PCOS. Further investigation is needed to clarify the link between leptin and IR in women with PCOS.

Resistin: First found by Steppan *et al*[209], resistin is an adipokine that exerts an inhibitory effect on adipocyte differentiation and exerts resistance to insulin in mice. It has been suggested that resistin could be the potential link between obesity and diabetes[209,210]. Moreover, resistin seems to be an important adipokine that is involved in obesity, IR and PCOS[211].

However, these are hypotheses that need to be ascertained in humans. Data regarding the association between resistin and IR remain controversial. Many studies failed to recognize any association between resistin and IR[212-213], while a few studies indeed discovered a significant positive correlation[214-215].

In women with PCOS, conflicting results have also been reported[216-218]. Munir *et al*[216], reported increased concentrations of serum resistin levels in women with PCOS in comparison to controls. However, no significant difference was found in circulating resistin levels between PCOS and controls in most studies[217,218].

Vaspin: Elevated serum and omental adipose tissue levels of visceral adipose tissue-derived serine protease inhibitor (vaspin) in overweight PCOS women and *ex vivo* regulation of vaspin, predominantly by glucose, were reported, for the first time, by Tan *et al*[219]. A similar result was found by Dogan *et al*[220]. However, Franik G *et al*[221], did not observe correlations between plasma vaspin levels and serum glucose and insulin concentrations or HOMA-IR values.

Apelin: Apelin is a peptide expressed in several organs and in visceral and subcutaneous tissues[222].

Controversial results have been reported by different authors. Several authors reported elevated apelin, while others reported low serum levels of the same[223-228]. Polak *et al*[8], in their recent review of the literature, concluded that discrepant findings among the published studies may be attributed to the differences in ethnicity, age, study design, sample size, genetic characteristics of populations, and assessment methodology. Further studies are necessary to elucidate the role of apelin in insulin resistance in PCOS.

Copeptin: Copeptin, a vasoactive peptide, has been reported to play an important role in CVD and metabolic disorders. Enhanced copeptin levels in PCOS patients are positively associated with fasting insulin, HOMA-IR, androgenic profile, triglycerides and carotid intima media thickness, indicating that copeptin may play an important role in cardiometabolic consequences in PCOS[8,229-231].

However, to date, few studies have been performed to assess copeptin as a marker of IR in women with PCOS.

Further data from large-scale longitudinal studies are required for its validation.

Irisin: Irisin is a myokine identified as a new marker of IR[8,232-234].

In PCOS, a significant positive correlation between circulating irisin, IR and dyslipidemia has been found. Li *et al*[233], demonstrated that irisin levels were significantly higher in PCOS subjects than in controls, as well as in overweight and obese patients than in lean women. Similar results were obtained by Li *et al*[234].

Further studies are necessary to confirm these findings.

Zinc-α2-glycoprotein: Zinc-α2-glycoprotein (ZAG) has been proposed to play a role in the pathogenesis of insulin resistance[235].

In women with PCOS, Lai *et al*[236], found that women with PCOS and high ZAG had fewer metabolic syndrome, IGT and polycystic ovaries than those with low ZAG. Taken together, circulating ZAG levels are reduced in women with PCOS. They concluded that ZAG may be a cytokine associated with insulin resistance in women with PCOS[236,237]. Pearsey *et al*[238], arrived at a similar conclusion.

Zheng *et al*[238], performed a study to investigate changes in ZAG levels after exenatide or metformin treatment. The results showed that circulating ZAG was significantly lower in women with PCOS than in healthy women. After 12 wk of exenatide or metformin treatment, there were significant increases in circulating ZAG in both treatment groups[238].

Therefore, more research is needed before robust conclusions can be drawn[8].

Plasminogen activator inhibitor-1: Numerous studies have reported the association between IR and plasminogen activator inhibitor-1 (PAI-1), a glycoprotein involved in the coagulation system[8,239-241].

PAI-1 has been found to be linked to insulin resistance in PCOS subjects[8,239-242].

Further data from large-scale longitudinal studies are required for its validation.

CONCLUSION

This article is an attempt to summarize existing markers of IR and their usefulness in women with PCOS. There is no recommended screening method for assessing IR in women with PCOS despite evidence of the high prevalence of this metabolic disturbance.

A host of methods have been described for assessing insulin resistance. Each method has its own merits and disadvantages.

The euglycemic clamp remains the gold standard for direct measurement of insulin sensitivity.

Concerning anthropometric surrogate markers, wrist circumference could revolutionize the assessment of IR in women with PCOS if validated through large-scale studies.

Regarding biological surrogate markers, HOMA-IR is the best and extensively validated marker.

Biological markers using lipids and lipoproteins are inconsistent in the Sub-Saharan African population and hence in Sub-Saharan African PCOS women.

Conflicting data concerning emerging markers in women with PCOS limit their use in the clinical setting.

Finally, an easy-to-detect marker for assessing IR in women with PCOS is urgently required.

FOOTNOTES

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REVIEW

Efficacy of probiotics on the modulation of gut microbiota in the treatment of diabetic nephropathy

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Abstract

Diabetic nephropathy (DN) is a major cause of end-stage renal disease, and therapeutic options for preventing its progression are insufficient. The number of patients with DN has been increasing in Asian countries because of westernization of dietary lifestyle, which may be associated with the following changes in gut microbiota. Alterations in the gut microbiota composition can lead to an imbalanced gastrointestinal environment that promotes abnormal production of metabolites and/or inflammatory status. Functional microenvironments of the gut could be changed in the different stages of DN. In particular, altered levels of short chain fatty acids, D-amino acids, and reactive oxygen species biosynthesis in the gut have been shown to be relevant to the pathogenesis of the DN. So far, evidence suggests that the gut microbiota may play a key role in determining networks in the development of DN. Interventions directing the gut microbiota deserve further investigation as a new protective therapy in DN. In this review, we discuss the potential roles of the gut microbiota and future perspectives in the protection and/or treatment of kidneys.

Key Words: Diabetic nephropathy; Short chain fatty acids; Superoxide dismutase; Reactive oxygen species; D-amino acids; Gut microbiota; Diabetes mellitus; Renal disease

Core tip: Evolving evidence suggests that the gut microbiota may play a key role in the development of diabetic nephropathy (DN). Interventions aimed at the gut microbiota deserve further investigation as a novel protective therapy in DN. We review the potential roles of the gut microbiota in the protection of kidneys and in the development of DN.

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INTRODUCTION

Diabetic nephropathy (DN) is a chronic disorder occurring in nearly 40% of patients with diabetes[1]. DN is an important cause of end-stage renal disease and a micro-vascular complication of diabetes mellitus (DM)[2,3]. Some dietary factors might be involved in the increase in renal failure in association with DM, showing that the number of patients with DN and/or DM has been increasing in Asian countries because of westernization of dietary lifestyle[2,3]. Pathogenesis of DN may be multifactorial and complex. Early DN has no noticeable clinical symptoms, however, hyperglycemia may be a significant risk factor for DN and/or DM[4]. Sustained elevated blood glucose could lead to changes in the downstream transcription factors and/or gene expression in kidney glomerular cells[5]. Kidney fibrosis and albu-minuria are key pathological processes of the advanced stage of DN[6], but oxidative stress and/or inflammation may also be important mechanisms for the pathogenesis of DN[7]. In general, oxidative stress and inflammatory responses are almost not distinct, because one reaction would intensify the other pathogenesis. Both DM and chronic kidney disease (CKD) may have a common pathophysiological mechanism within a chronic inflammatory state and/or oxidative stresses [8]. Among them, high levels of reactive oxygen species (ROS) could induce inflammatory cytokines in the kidney[9], which might accelerate the development of DN. Inflammation of the kidneys can lead to proteinuria and/or persistent hypertension, which can proceed to renal failure. Hence, successful treatment of the microcirculation in patients with DN has become a superior strategy for the prevention of DN. This reasonable treatment should be discovered immediately. Recently, it has been shown that pathogenesis of DN is associated with certain gut microbiota[10]. The importance of probiotics is widely recognized in various diseases. Besides, studies have shown that crosstalk between host and microbiota might be relevant pathologically in patients with DN[11]. For example, alterations in the gut microbiota are associated with the development of proteinuria[12], and type 2 DM[13]. Changes to the gut microbiota have also been reported in DM and DN[14]. The gut microbiota might well communicate with the kidneys, and the collapse of this relationship might result in the development of renal dysfunction. Accordingly, the gut microbiota could be an important defense against the pathogenesis of kidney disease. Dietary lifestyles have radically changed over the last century in developed countries, and are characterized by reduced dietary fiber and/or increased high-fat consumption[15]. Hence, the changes could be linked to alteration of gut microbiota[16]. Abnormal intestinal metabolites and disruption of the intestinal barrier owing to the gut dysbiosis might facilitate harmful substances produced in the gut entering the circulatory system[17]. These situations allow us to hypothesize that dietary changes could lead to a microbiome that modifies positively the threshold and/or the speed of developing DN and/or DM.

GUT-KIDNEY AXIS IN THE PATHOGENESIS OF DN

Although the significance of the gut microbiota has yet to be completely determined, it is obvious that an intricate symbiotic relationship might exist between host and microbe. In addition, the interaction has recently attracted interest in the study of the pathogenesis of various disorders. The human body holds numerous bacterial and/or microbial cells; the majority of which exist in the gut[18]. The microbiota is a complex community of more than 100 trillion cells in healthy human intestines[19]. The normal gut microbiota could protect the kidney, whereas gut dysbiosis of the microbiota could facilitate kidney disorders[20]. Furthermore, alterations in the microbiota are gradually being linked to the development of various other diseases such as inflammatory bowel disease, cancer, psychiatric disorder, and cardiovascular disease[21]. The gut-kidney axis could additionally affect metabolic and/or immune pathways in addition to the related diseases[22]. The gut-kidney axis is largely mediated by metabolites produced by the gut microbiota, which might regulate physiological function of several organs including the brain, pancreas, adrenal glands, kidneys, *etc.* (Figure 1). For example, components of the immune system might have a key role with cytokines in communication between the gut and kidneys [23]. Furthermore, crosstalk between the metabolic and immune pathways has a significant role in keeping a good balance in the kidneys[23]. Intestinal responses to inflammation and/or infections are intricate. If microbiota-immune pathways overstimulate tolerance to some inflammation, greater inflammation may accelerate progression of renal disease and/or its complications. Accordingly, gut dysbiosis has frequently been associated with progression of many kidney diseases[24]. In addition, accumulation of uremic toxins, which are derived from dietary metabolism in the gut and/or liver, has distinct effects

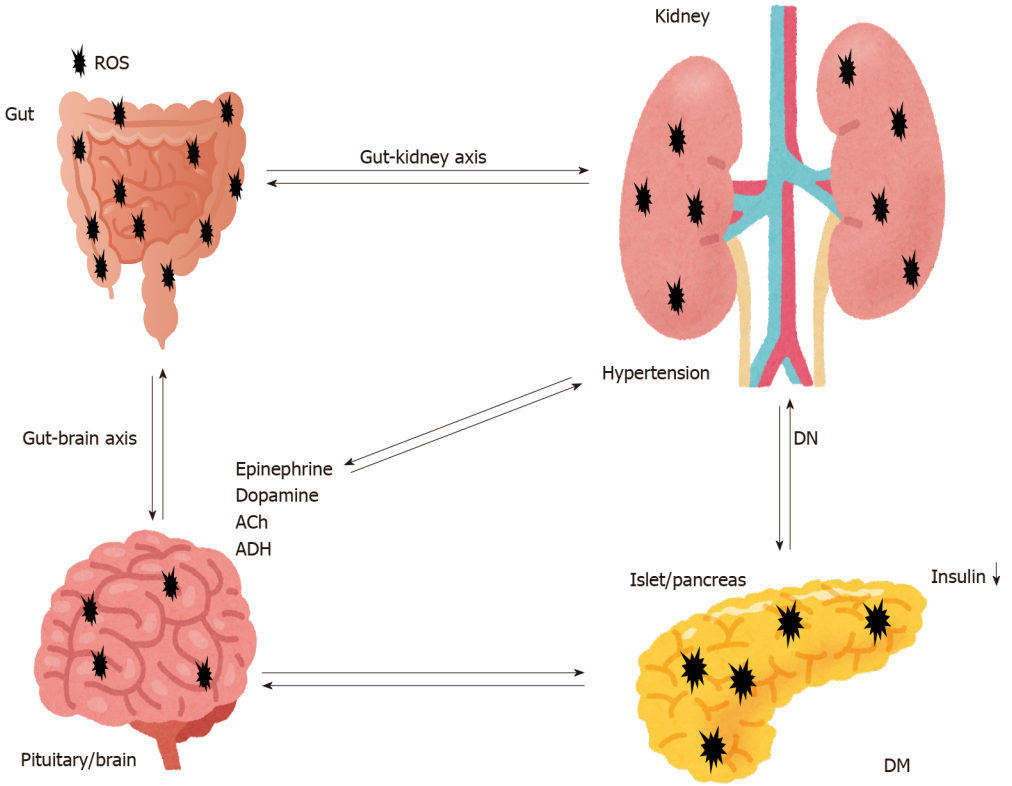

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Figure 1 Representation of the pivotal role of gut-kidney axis crosstalk with the brain and the pancreas in the pathogenesis of diabetic nephropathy. Hypothetical image of the pathogenesis pathway for diabetic nephropathy (DN). Sympathetic activation is a common feature in disorders of the brain as well as gut and kidneys. The brain is responsible for sympathetic outflow contributing to an increase in blood pressure and pathogenesis of the gut and kidneys. Dysbiosis in the gut results in an imbalance of intestinal homeostasis. Pathological events in the brain, pancreas, adrenal glands, gut and kidneys significantly contribute to the development of hypertension and DN. Note that some critical pathways such as inflammation pathway have been omitted for clarity. Ach: Acetylcholine; ADH: Antidiuretic hormone; ROS: Reactive oxygen species; DM: Diabetes mellitus; DN: Diabetic nephropathy.

on the kidneys. For example, the increase in urea increases its influx into the bowel lumen from epithelial cells, where it is hydrolyzed by gut microbiota urease to ammonia[25]. Subsequently, ammonia byproducts may increase the bowel pH, leading to the severe mucosal damage[26]. Accumulation of the uremic toxins in combination with inflammation may also increase the risk of renal disease [27]. Therefore, key factors in kidney disease are function of the gut microbiota and/or the action of gut dysbiosis. Inflammatory bowel disease and DM are indeed multifactorial diseases, and both are chronic diseases associated with increased risk of various diseases including cardiovascular disease, which indicates that the gut is associated with host physiological functions[28]. Interestingly, the prevalence of inflammatory bowel disease in adults with type 1 DM is higher compared to that of nondiabetic controls [29]. It is plausible that the gut-kidney axis might be involved in the pathogenesis of inflammatory bowel disease and DM. Similarly, the gut microbiota may be involved in the damage of other organs, hence targeting the gut microbiota could represent a future therapeutic approach in various diseases. However, the potential impact of gastrointestinal-related disorders on the development and/or progression of DN remains to be elucidated.

LEVELS OF SHORT-CHAIN FATTY ACIDS, ROS, AND D-AMINO ACIDS MAY BE INVOLVED IN THE DEVELOPMENT OF DN

Diabetic model mice fed with a high-fiber-diet are less likely to develop DN compared with diabetic control mice fed with a no-fiber diet[30]. High-fiber diet might decrease the expression of genes encoding inflammatory cytokines related to DN[30]. In general, fibers positively improve the dysbiosis of microbiota with promoting the production of short chain fatty acids (SCFAs) (including butyrate, acetate and propionate) in gut microbiota[31], which might also increase the production/release of cytokines and/or chemokines[32]. In addition, SCFAs are able to inhibit intestinal inflammation and/or oxidative stress[33]. Major SCFAs (acetate, propionate and butyrate) are derived through glycolysis of glucose to pyruvate or acetyl-CoA. The SCFAs regularly induce glucagon-like peptide 1 secretion through stimulation of a G-protein-coupled receptor (GPCR)[34]. Gut microbiota in older people may

weaken SCFA production[35]. Those SCFAs have various effects on endocrine cells in gut *via* the GPCRs such as G-protein-coupled receptor (GPR)43 or GPR109A[36]. SCFA-treated diabetic mice have been shown to be protected from nephropathy, suggesting that SCFAs protect renal cells from injury by oxidative stress in DN[37]. It has been shown that butyrate, one of the SCFAs produced by gut microbiota, plays a protective role in DN, which contributes in various physiological processes predominantly by inhibiting histone deacetylases (HDACs)[38]. In addition, providing sodium butyrate has been shown to protect renal cells from oxidative damage and/or apoptosis in type 2 DN mice[39]. Consistently, sodium butyrate has inhibited high-glucose-induced apoptosis of tubular epithelial cells in normal kidneys[40]. Sodium butyrate also lowers plasma glucose and nuclear factor-B expression in the kidneys and attenuates kidney injury[41]. In experimental mice, suppression of HDACs by sodium butyrate may explain the decrease in apoptosis in the kidneys[42]. HDACs can regulate cell proliferation, migration and apoptosis, which are organized by a family of enzymes important for chromatin remodeling, keeping a dynamic balance with histone acetyltransferases in expression of several genes [43]. Valproate, an HDAC inhibitor, has also been shown to decrease renal injury and/or renal fibrosis [44].

The signaling pathways triggered by hyperglycemia appear to have a pivotal role in diabetic complications due to the production of ROS and/or additional oxidative stress, which finally leads to apoptotic cell death in various tissues[45]. ROS includes superoxide anions, hydroxyl free radicals, and hydrogen peroxide[46]. The mitochon-drial electron transport chain is considered a major endogenous source of ROS[47]. Production of excess ROS leads to increased membrane permeability and serious cellular damage[48]. Such overproduction of ROS links to the pathological condition of altered metabolic pathways in the kidneys and disturbed renal function known as nephropathy[49]. Once ATP synthesis is dysregulated in this hyperglycemic situation, it can result in excess production of ROS, which leads to kidney failure[50]. Furthermore, high glucose exposure with excessive ROS can lead to renal podocyte apoptosis in experimental DN[51]. Antioxidants including ubiquinone (also termed coenzyme Q10), ascorbic acid, and resveratrol have been tested in animal models of kidney diseases with some evidence of therapeutic benefits[52]. Epidemiological studies have also found an association between high levels of ROS and risk of DN[53]. Therefore, downregulation of ROS and/or oxidative stress might have a crucial role in regulating diabetic complications. Besides, ROS have been revealed to function as second messengers in several signal transduction pathways[54,55].

Studies have shown the clinical significance of D-amino acids in several kidney diseases[56]. For example, the combination of blood level and urinary dynamics of D-serine effectively separates CKD from non-CKD[57]. D-amino acids in body fluids are also a promising early detection marker for kidney disease[58]. However, excess D-serine can cause kidney damage in rats[59]. In this case, it has been shown that D-serine administration can initiate extensive necrosis in renal proximal tubules[59]. In contrast, administration of D-alanine does not induce kidney injury[60]. Furthermore, protective effects of low-dose D-serine have likely been shown to suppress renal damage, which may promote the hypoxia-mediated proliferation of tubular epithelial cells[61]. In addition, D-cysteine administration can also protect the kidneys from ischemia-reperfusion injury, which might be useful to treat various renal diseases[62]. D-aspartate plays a role during development and neurogenesis[63]. D-aspartate treatment might produce favorable effects during demyelination and remyelination in the nervous system[64]. Furthermore, the ovary-inducing activity of D-tryptophan is more effective than that of L-tryptophan [65]. These data suggest that D-amino acids have both beneficial and harmful effects on tissue development and/or tissue-protection (Figure 2).

GUT MICROBIOTA COULD CONTRIBUTE TO HEALTHY KIDNEYS

Carbohydrates are metabolized by gut bacteria into monosaccharides and oligosaccharides, and they could be fermented into SCFAs. As shown above, SCFAs are one of the primary end products of gut fermentation that have considerable effects on host physiology. SCFAs can act as signaling molecules between the gut microbiota and host, and may have a protective effect on the renal function of patients with CKD. In particular, butyrate improves the intestinal barrier and reduces lipopolysaccharide influx into the blood, which could attenuate progression of DN[66]. We provide here a perspective of gut-kidney axis applied in search of renal disease management associated with the gut microbiome, which may theoretically be beneficial for future treatment of DN. Diet is known to be an essential regulator of gut microbiomes[67]. Many studies have confirmed the association between nutrition and the human microbiome in maintaining human health, suggesting significant roles of bacterial metabolites in both health and disease[68]. Trillions of bacteria present in the intestinal and colon lumina constitute the human gut microbiota[69]. Dietary intake could control microbiota whose fermentation may produce various metabolites including SCFAs[70]. The metabolites might additionally regulate the growth of pathogens by competing for of nutrients. For example, parenteral nutrition has been associated with a change in the microbiota, altering SCFA production, and inducing gut mucosal atrophy[71]. The SCFAs made by the healthy gut microbiota have anti-inflammatory properties, including proliferation of regulatory T cells[72,73]. In addition, a significant role for regulatory T cells has been revealed in type 2

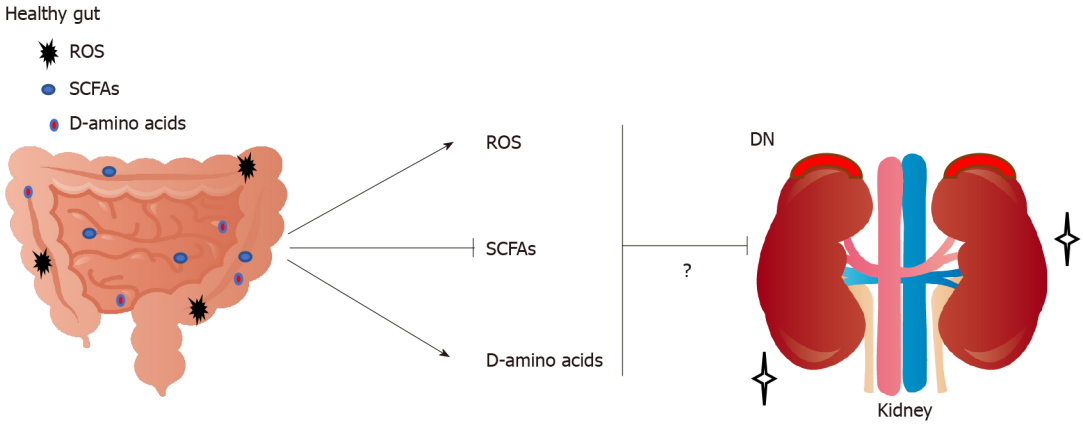

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Figure 2 Implication of increased short-chain fatty acids, decreased reactive oxygen species, and increased D-amino acids derived from gut in the renal protection and/or exacerbation against the progression of diabetic nephropathy. Arrowheads mean stimulation and/or progression, whereas hammerhead represents inhibition. Note that some critical pathways including hormonal regulation have been omitted for clarity. SCFAs: Short-chain fatty acids; ROS: Reactive oxygen species; DN: Diabetic nephropathy.

diabetes for protection against DN[74]. In addition, SCFAs have favorable effects on β cells, potentiating glucose-stimulated insulin release and/or maintaining β-cell mass through inhibiting apoptosis[75]. Furthermore, propionate, has been shown to prevent adipogenic differentiation of specific stem cells [76].

Many studies have emphasized the relationship between the gut microbiota and oxidative stress[77]. In general, ROS production has a defense mechanism that could elicit cytotoxicity against several pathogens then reduce the burden of infection[78]. Redox signaling is also found in response to microbial signals *via* the gut epithelial NADPH oxidase 1[79]. Therefore, microbial ROS might rigorously control signaling processes for appropriate immunity and/or the gut barrier[80]. Numerous bacterial species of the microbiota can reduce mitochondrial ROS production[81]. For example, microbial products can upregulate the activity of superoxide dismutase, which results in reduced ROS levels and then decreased cellular apoptosis[82]. In addition, microbial excess ROS might disturb other important pathways of host cells, suggesting that ROS-mediated signaling can regulate various cellular processes in order to keep the host healthy[83]. Epithelial cells may also exhibit increased ROS production in response to several harmful bacteria[84]. In the gut, epithelial appropriate ROS production in response to the gut bacteria may play a signaling role in the host[85]. It is likely that there are many ROS-sensitive important enzymes that could be affected by alterations in the gut redox conditions.

Finally, the gut microbiota have the largest genetic capacity to metabolize D-amino acids that are utilized as nutrients to support bacterial growth to regulate spore germination[86]. Therefore, one possible source of D-amino acids in mammals may be their gut microbiota. In general, many bacterial species encode racemases that convert L-amino acids to D-amino acids[87]. For example, D-alanine production is associated with a relative abundance of bacterial species with racemases such as those of *Enterococcus* and *Lactobacillus* in the gut microbiota[88]. Different bacterial species may produce distinct profiles of D-amino acids[89]. Higher D-amino acids levels have been related to the gut microbial mass [90]. Oral intake of a peptide containing specific D-amino acids may reverse the diabetes-associated pathological alterations in the kidneys[91] (Figure 2). Noteworthy differences in the microbiota composition have been discovered in patients with kidney disease compared with healthy controls[92]. Consequently, treatment options for DN should include dietary therapy affecting the gut microbiota. Therapeutic interventions would nevertheless represent a potential target of the microbiota for prevention and/or treatment of DN.

CONCLUSION

New therapies for DN are emerging. One method that may affect the gut microbiota composition is fecal microbiota transplantation (FMT) (Figure 3). The beneficial effects of the transplantation are dependent on the host responses, however, which may provide a potential treatment strategy for type 2 diabetes[93]. In particular, transplantation of *Faecalibacterium prausnitzii* (*F. prausnitzii*) could restore the intestinal structure, which might be used as a potential therapeutic approach against inflammation as well as diabetes[94-96]. Furthermore, *F. prausnitzii* may serve as a diagnostic and therapeutic biomarker for the use of FMT[97]. The potential role of the gut microbiota has been hypothesized to modulate renal function in experimental DN murine models[98]. Through FMT, the role of the gut microbiota and its

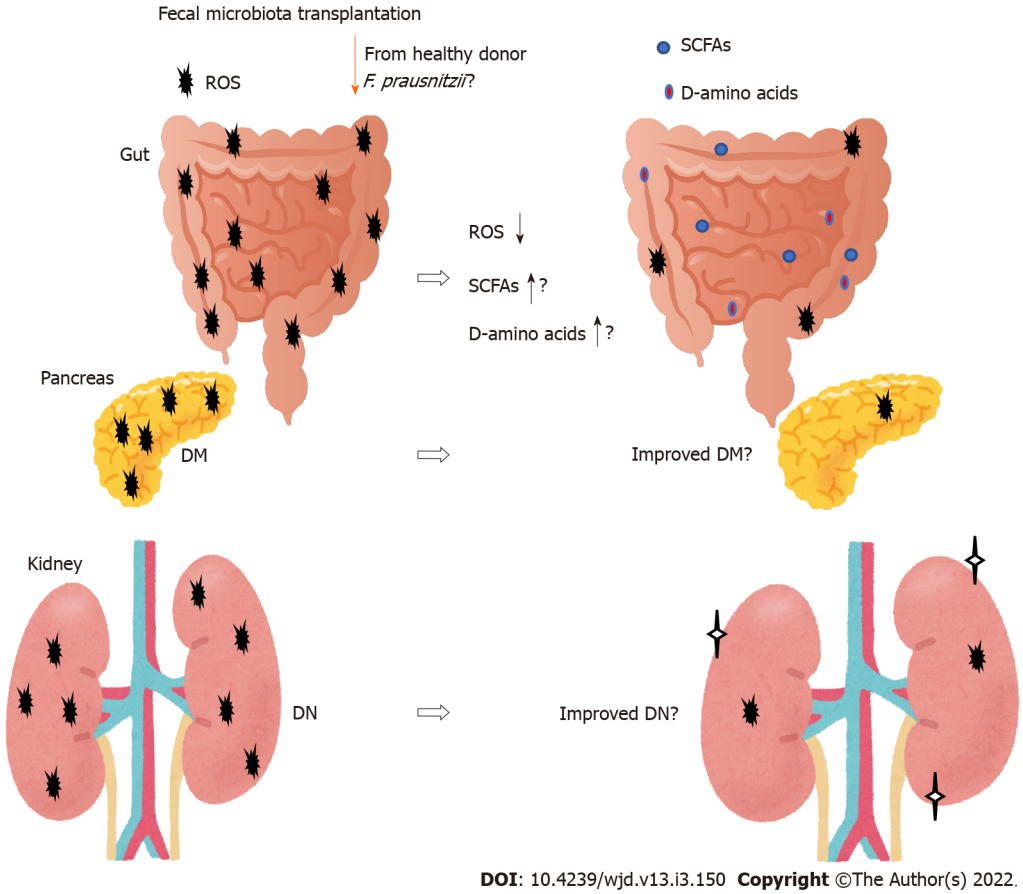

Figure 3 The gut microbiota could contribute to the favorable production of short-chain fatty acids, reactive oxygen species and D-amino acids against progression of diabetic nephropathy. Fecal microbiota transplantation consists of fecal microbiota infusion from a healthy donor to a recipient, which has been likely more successful than conventional therapy for diabetic nephropathy. Note that some critical events such as cytokine-induction have been omitted for clarity. SCFAs: Short-chain fatty acids; ROS: Reactive oxygen species; DN: Diabetic nephropathy; DM: Diabetes mellitus.

SCFA production have been verified in the treatment of DN. Therefore, administration of prebiotics and/or probiotics should individually be tailor-made to prevent and/or cure chronic diseases such as DN. For example, acetate produced by certain gut microbiota reprogramming has been shown to contribute to the tubulointerstitial injury of DN, suggesting that gut microbiota might be a new strategy for DN treatment[99]. Furthermore, FMT from healthy donors considerably attenuates glomerular injury with podocyte improvement in diabetic rats[100].

The above-mentioned topics are only just being explored in preclinical research, suggesting that further studies are required. Owing to a lack of treatments, DN has been a public health concern. Although it is untimely to draw definitive conclusions about the clinical usefulness of microbiota-based treatment strategies for DN, modulation of gut microbiota is an exciting frontier in kidney research. It is clear that intensive evaluation of preclinical studies is necessary to find further insights. In addition, long-term studies are also necessary to clarify the detailed effects of probiotic treatment in the management of DN. A healthy lifestyle with a balanced familiar diet is now one of the main recommendations.

FOOTNOTES

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REVIEW

Protective effects of physical activity against health risks associated with type 1 diabetes: "Health benefits outweigh the risks"

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Abstract

The magnitude of diabetes mellitus (DM) has increased in recent decades, where the number of cases and the proportion of the disease have been gradually increasing over the past few decades. The chronic complications of DM affect many organ systems and account for the majority of morbidity and mortality associated with the disease. The prevalence of type 1 DM (T1DM) is increasing globally, and it has a very significant burden on countries and at an individual level. T1DM is a chronic illness that requires ongoing medical care and patient self-management to prevent complications. This study aims to discuss the health benefits of physical activity (PA) in T1DM patients. The present review article was performed following a comprehensive literature search. The search was conducted using the following electronic databases: "Cochrane Library", Web of Science, PubMed, HINARI, EMBASE, Google for grey literature, Scopus, African journals Online, and Google Scholar for articles published up to June 21, 2021. The present review focused on the effects of PA on many outcomes such as blood glucose (BG) control, physical fitness, endothelial function, insulin sensitivity, well-being, the body defense system, blood lipid profile, insulin resistance, cardiovascular diseases (CVDs), insulin requirements, blood pressure (BP), and mortality. It was found that many studies recommended the use of PA for the effective management of T1DM. PA is a component of comprehensive lifestyle modifications, which is a significant approach for the management of T1DM. It provides several health benefits, such as improving BG control, physical fitness, endothelial function, insulin sensitivity, well-being, and the body defense system. Besides this, it reduces the blood lipid profile, insulin resistance, CVDs, insulin requirements, BP, and mortality. Overall, PA has significant and essential protective effects against the health risks associated with T1DM. Even though PA has several health benefits for patients with T1DM, these patients are not well engaged in PA due to barriers such as a fear of exercise-induced hypoglycemia in particular. However, several effective strategies have been identified to control exercise-induced hypoglycemia in these patients. Finally, the present review concludes that PA should be recommended for the management of patients with

T1DM due to its significant health benefits and protective effects against associated health risks. It also provides suggestions for the future direction of research in this field.

Key Words: Type 1 diabetes mellitus; Physical activity; Health benefit; Glycemic control; Exercise

Core Tip: Diabetes mellitus (DM) is a group of chronic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The impairment of beta-cell function is an ancient feature of disease pathogenesis, while a significant reduction in beta-cell mass is closely associated with clinical manifestations in type 1 DM and type 2 DM. Physical activity (PA) is good for almost every individual. PA is a significant mediator of glycemic control and prevents pathologies related to increased postprandial glucose. Its significant role in the prevention and management of noncommunicable diseases is extensively understood. PA is widely known to be an effective approach for the prevention and management of numerous chronic diseases.

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INTRODUCTION

Diabetes mellitus (DM) is a group of chronic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both[1]. The loss of beta-cells (β-cells) is a determinant factor for the development of type 1 DM (T1DM)[2]. In T1DM and type 2 DM (T2DM), the impairment of β-cell function is an early feature of disease pathogenesis, while a significant reduction in β-cell mass is closely associated with clinical manifestations[3]. Reduced functional β-cell mass is the hallmark of both T1DM and T2DM and this triggers absolute or relative insulin insufficiency in both circumstances [4]. T1DM is characterized by an immune-mediated depletion of β-cells that results in a lifelong dependence on exogenous insulin[5-9]. Despite all the efforts made to identify efficient therapeutic methods for T1DM, insulin is the only effective treatment[2].

However, even modest levels of β-cell activity were associated with a reduction in the incidence of retinopathy and nephropathy in T1DM individuals[10]. Even though the factors that predict the occurrence and rapidity of the decrease in β-cell function are still largely unknown, evidence has identified islet cell autoantibodies as predictors. Historical as well as recent clinical experience has underlined the significance of residual insulin production for glycemic control and to avoid end-organ complications[11]. T1DM can arise at any age while a peak in incidence is seen around puberty[12]. Overweight and obesity are highly prevalent among young people and adults with T1DM which is (25%–35%) and (37% to nearly 80%), respectively. Obesity raises the risk of developing T1DM and may lead to an earlier age at diagnosis. Also, obesity may raise the risk of macrovascular disease, retinopathy, and metabolic syndrome among these patients[13]. The chronic hyperglycemia of diabetes is linked to long-term damage, dysfunction, and failure of different organs, particularly the eyes, kidneys, nerves, heart, and blood vessels[1].

Generally, diabetes-related complications can be divided into macrovascular and microvascular complications. Stroke, coronary artery disease, and peripheral arterial disease are included under macrovascular complications and microvascular complications comprise, retinopathy, diabetic nephropathy, and neuropathy[14]. In addition, T1DM is linked with premature cardiovascular disease (CVD)[15]. In T1DM individuals, the absolute and relative risks of CVD remain very high[16]. When women with T1DM are compared to men with T1DM, women have nearly 40% more excess risk of all-cause mortality and twice the excess risk of fatal and nonfatal vascular events[17]. The age at onset and the duration of T1DM seem to be significant determinants of survival and all cardiovascular outcomes. Early onset is associated with up to a 30-fold augmented risk of serious cardiovascular outcomes, with risk levels being 90-fold greater for women with early-onset diabetes, and who die approximately 18 years earlier than nondiabetics[18]. Vascular complications are a significant cause of morbidity and mortality in individuals with T1DM and T2DM[19].

The magnitude of DM has increased in recent decades[20], where the number of cases and the proportion of the disease have been gradually increasing over the past few decades[21]. The incidence of childhood T1DM is significantly increasing globally[22]. It is the epidemic of the century and without effective diagnostic approaches at an early stage, diabetes will continue to increase[23]. The global

estimates for the prevalence of diabetes for 2015 and 2040 were 8.8% and 10.4%, respectively[24]. Whereas it was 8.8% and 9.9% of the world population in 2017 and by 2045, respectively[25].

In recent decades, a significant increase in the proportion of DM has been evidenced in nearly all regions of the world. The increase in the number of subjects with the disease is possibly due to a change in the disease profile in numerous populations around the world and this is primarily because of a larger incidence of diabetes-related complications such as kidney failure and peripheral arterial disease [26]. It is a significant public health problem and is one of the four ranked noncommunicable diseases targeted for action by world leaders[21]. T1DM is associated with an increased risk of CVDs and all-cause mortality in insulin-treated patients with diabetes while the connection between hypoglycemia and cardiovascular consequences and mortality exists over a long period[27]. Whereas, more than the general population, elderly individuals with diabetes have higher all-cause mortality rates[28]. The excess mortality observed in T1DM is almost totally associated with diabetes and its related comorbidities[29].

The increasing disease burden of DM globally is a major public health priority, placing a fluctuating need on the patients, their careers, health systems, and society[30]. Diabetes is one of the leading and rising causes of hospital admission and disability due to other diseases[31]. This high magnitude of the disease has a greater social and financial burden[24]. It imposes a rising economic burden on national health care systems globally[32]. The global costs of DM and its significance are huge and will markedly rise by 2030[33]. Even though the present data found an increase in the magnitude of diabetes, the recent understanding of the international burden of and variation in the disease linked with complications is poor worldwide[26].

Diabetes is a chronic illness that requires ongoing medical care and patient self-management to prevent complications. Diabetes care is complex, requires numerous issues to be addressed, and it is more than glycemic control[34]. T1DM is a chronic disease with severe complications due to its mismanagement. The health professionals should be equipped with suitable evidence based on multiple management approaches to those individuals to support patient-centered care and improve their capacity for problem-solving and self-management[35]. Maintaining the long-term integration of lifestyle changes and medical management is crucial to accomplish good metabolic control in diabetes subjects[14,36].

Self-management participation could lead to clinically associated progress in the behavior and clinical parameters[37] and those individuals who participate in self-management can be considered volunteers in the majority of cases where they have either wanted an intervention or decided to take part[38]. Physical activity (PA) and nutrition are significant components of a healthy lifestyle and treatment of diabetes[39]. The benefit of exercise in T1DM remains a significant component of its treatment[40]. Therefore, the adoption and maintenance of PA are crucial for the management of glycemia and the entire health of individuals with diabetes and prediabetes[41]. Exercise is a cornerstone in the lifestyle of nearly all individuals with T1DM[42]. As the patients may be more agreeable to lifestyle changes, exercise should be encouraged from diagnosis. In addition, to improve patient confidence in managing their diabetes with exercise, standard advice on exercise and diabetes needs to be made available to health professionals and subjects with diabetes[43].

With regard to PA, even though, the term PA and exercise are not synonymous they are often used interchangeably[44,45]. However, the term "PA" should not be mistaken with "exercise", as exercise is a subgroup of PA[46]. Due to this, it is recommended that they should not be used interchangeably[47]. PA can be defined as any bodily movement formed by the skeletal muscles that result in energy expenditure above resting levels[46,48-50]. While exercise is defined as a planned, structured PA typically performed with the intent of improving health and/or fitness[46,48]. The term PA is broadly comprised of exercise and sport, and PA is performed as a part of daily living, occupation, leisure, and active sport[46,48]. Exercise can be classified as aerobic and resistance exercise[51]. Aerobic exercise involves the repeated and continuous movement of huge muscle groups[52]. Anaerobic exercise comprises activities such as walking, cycling, jogging, and swimming. Resistance exercise includes activities such as free weights, weight machines, bodyweight, or elastic resistance bands[51].

METHODS

Research questions

What are the protective effects of physical activity against the health risks associated with T1DM?

Study setting

The present review article includes all studies conducted in various countries globally.

Search strategies

The present review article was carried out using a comprehensive literature search. The search was performed using the following electronic databases: “Cochrane Library”, Web of Science, PubMed, HINARI, EMBASE, Google for grey literature, Scopus, African journals Online, and Google Scholar. The

search was conducted using the following search terms; "diabetes mellitus", "type 1 diabetes", "T1DM", "complications", "insulin-dependent diabetes mellitus", "IDDM", "physical activity", "exercise", "Glycemic Control", "Physical Fitness", "Blood Lipids Profile", "Endothelial Function", "Insulin Resistance", "Insulin Sensitivity", "Insulin Requirement", "Cardiovascular Diseases", "Blood Pressure", "Well-being", "Body's Defense Systems", "Mortality", "barriers", "factors", "strategy", and "hypoglycemia". The Boolean operators; "AND" and "OR" were used to integrate them during the search.

Eligibility criteria

The inclusion criteria for the present review were; Articles on this topic globally, published in the English language, quality articles, and those with outcome variables well defined and measured, and articles published up to June 21, 2021. The exclusion criteria for the present review article were: articles of poor quality and articles in which the outcome variable was not clearly defined and measured.

THE HEALTH BENEFITS OF PHYSICAL ACTIVITY FOR T1DM PATIENTS

Overall, PA is good for almost every individual[52]. It provides numerous health benefits mainly for obese individuals[53]. Even among healthy individuals, daily PA is a significant mediator of glycemic control and enhances the prevention of pathologies related to increased postprandial glucose[54]. Its significant role in the prevention and management of noncommunicable diseases is extensively understood[55]. Exercise is largely known as an effective approach for the prevention and management of many chronic diseases[56]. It is essential in the primary and secondary prevention of chronic diseases such as CVD, diabetes, cancer, hypertension, obesity, depression and osteoporosis, and premature death [57]. The effects of exercise also include the management of many metabolic syndromes as well as improved mood and quality of life[58].

Exercise training leads to improved body composition, cardiovascular, and metabolic outcomes in subjects with metabolic syndrome[59]. Moreover, PA helps to decrease all causes of morbidity and improves the quality of life in people of all age groups[60,61]. It also benefits principally older adults by protecting against and ameliorating several diseases, the achievement and maintenance of a healthy body weight, improved mental health and well-being, and musculoskeletal health. The amelioration of disease risk factors, the achievement of peak bone mass, and maintenance of healthy body weight were the benefits of PA in children[62].

Activity may play a protective role due to the consistent relationship between historical PA and the development of complications in insulin-dependent DM (IDDM)[63]. Exercise also has many significant health merits in both T1DM and T2DM subjects[64] and many exercises are supportive in these subjects [65]. The findings from randomized trials support the role of resistance training as an adjunctive mode of management in T1DM patients[66]. Both aerobic and resistance exercises are excellent for patients with T1DM[67]. In addition, regular moderate to vigorous PA was linked with many health benefits in adolescents with T1DM[68]. Exercise decreases the rate of diabetes-related complications in T1DM subjects[69].

Vigorous-intensity PA has a role in metabolic control in T1DM patients[70]. Consistent regular PA can improve metabolic control in these patients[71] and is significant for best physical and psychological development during childhood, and it improved glycemic control, cardiovascular function, blood lipid profiles, and psychological well-being[72]. Consistent PA has a beneficial effect on glycemic control, diabetes-related comorbidities, and cardiovascular risk factors without the risk of adverse events[73]. A summary of the effects of PA on many health outcomes such as blood glucose (BG) control, physical fitness, endothelial function, insulin sensitivity, well-being, body defense system, blood lipid profile, insulin resistance, CVDs, insulin requirements, BP, and mortality is shown in Figure 1.

GLYCEMIC CONTROL

Discrepancies have been observed in the literature regarding the role of PA in T1DM glycemic control. For instance, in T1DM female individuals, daily physical training for several months did not improve glycemic control[74]. In addition, Yki-Jarvinen *et al*[75] demonstrated that a controlled physical training program in pump-treated T1DM subjects did not change an already near-normal glycemic control. Furthermore, Zinman *et al*[76] found that, although plasma glucose declines acutely with exercise, an augmented caloric intake on exercising days obviates the long-term effect of training on glucose control. Similarly, glycemic control did not significantly improve in pregnant women with T1DM during postprandial walking exercise[77]. Moreover, glycemic control was not found to be associated with long-term PA in T1DM subjects and PA did not negatively affect long-term glycemic control[78].

Several studies have found that PA improved glycemic control in individuals with T1DM[69,71,79-113]. Regular PA can lead to decreased BG level among these patients, it is safe and does not result in

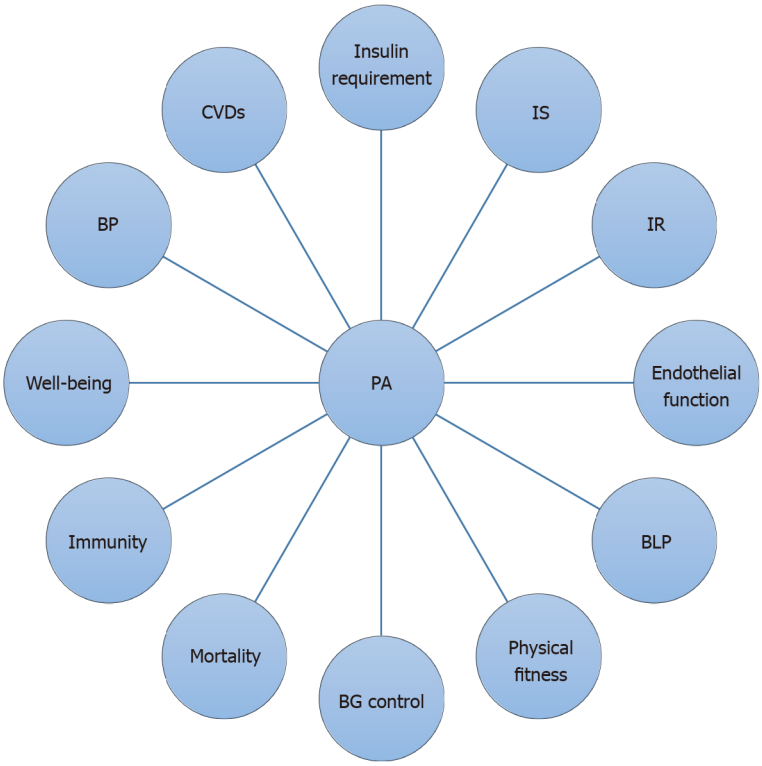

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Figure 1 The health benefits of physical activity in type 1 diabetes patients. PA: Physical activity; BG: Blood glucose; BLP: Blood lipid profile; IR: Insulin resistance; IS: Insulin sensitivity; CVDs: Cardiovascular diseases; BP: Blood pressure.

more hypoglycemic episodes[91]. A systematic review showed that PA had a positive impact on glycemic control in children and adolescents with T1DM[97]. Similarly, regular PA enhances BG control in children with T1DM[98]. Prolonged moderate aerobic exercise leads to a consistent decrease in plasma glucose but frequent hypoglycemia can occur when pre-exercise glucose concentrations are < 120 mg/dL in young people with T1DM[100]. Increased leisure-time PA (LTPA) between the ages of 50 and 70 years in the absence of active intervention was also found to be associated with improved glucose in men[101]. Campaign *et al*[71] demonstrated that regular high-intensity PA can improve metabolic control in young children with IDDM. In addition, combined exercise training (endurance training and resistance training) improves glycemic control to a better extent than endurance or resistance training alone, under moderate-intensive training situations with equal training durations [82]. Supervised strength training in T1DM male patients was associated with significant changes in glycemic control[93]. Marrone *et al*[95] found that free-play PA has a crucial role in helping to maintain BG levels in children with T1DM. Furthermore, anaerobic circuit training was found to improve glucose regulation in adolescents with IDDM[96]. Regular participation in moderate to intense PA or sports improves metabolic control in T1DM subjects[103]. High-intensity training (HIT)[105] and resistance training[106] improve plasma glucose in T1DM patients. Generally, an enhanced skeletal muscle, by either an intrinsic mechanism or PA, provides better advantages and benefits in facilitating glucose regulation[86] as peripheral glucose utilization rises during exercise, despite a reduction in circulating insulin levels[85]. During PA, muscle glucose uptake also rises and can reach values that are 30-50 times greater than at rest[87].

PA decreased glycosylated hemoglobin (HbA1c) in T1DM patients[81,89,90,111-113]. This effect is acceptable since the HbA1c level is increased following PA cessation[89]. This shows that the reduction of HbA1c level is a major sign of glycemic control. This is because the amount of glucose that combines with HbA1c is directly proportional to the total amount of glucose within a system. This means, if the BG levels have been high in current weeks, the HbA1c level will also increase. This could be evidence of PA reducing BG level, and was proved by the decrease in this biomarker of glycemic control.

PHYSICAL FITNESS

Physical fitness is defined as a set of attributes that are either health- or skill-related and the extent to which individuals have these attributes can be measured with specific tests[50]. Evidence shows that patients with T1DM have reduced physical fitness[114]. Furthermore, children with T1DM presenting

with poor glycemic control had lower aerobic fitness compared to those with good glycemic control [115]. In addition, lower cardiorespiratory fitness in children with T1DM is associated with poor glycemic control[116].

However, numerous studies have shown that PA improves physical fitness in individuals with T1DM [92,93,96,97,117,118]. Supervised strength training in male patients was associated with augmented strength[93]. Also, exercise training among adolescents with T1DM leads to improved physical fitness [92]. Even, a training program of 1 h per week for 3 mo was found to improve physical fitness[117]. Anaerobic circuit training improved muscle strength in adolescents with IDDM[96]. Furthermore, a systematic review showed that PA improved physical fitness in children and adolescents with T1DM [97]. Moreover, a randomized trial demonstrated that combined exercise training appeared to improve physical fitness in adolescents with T1DM[118]. Regular PA also improved cardiovascular fitness[71,98, 102,107] and increased lean mass in these patients[98].

BLOOD LIPIDS PROFILE

A study found that youths with T1DM have abnormal lipid levels and atherogenic changes in lipoprotein composition, even after a relatively short disease duration, and glycemic control is a significant mediator of these abnormalities[119]. In normal-weight T1DM youths, mainly females had more atherogenic low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) distributions which are associated with lower insulin sensitivity[120]. Dyslipidemia is significantly more frequent in children and adolescents with T1DM compared to non-diabetic peers whilst high LDL-C and low HDL-C were the most frequent type of dyslipidemia in the dyslipidemic group[121]. Similarly, dyslipidemia is frequently found in T1DM and appears to be associated with glycemic control. It is a major risk factor for coronary heart disease, and one of the most significant and frequent complications with a high premature mortality and morbidity rate[122]. In addition, apolipoprotein B is consistently associated with an increased risk of mortality in T1DM due to all causes as well as in cardiac disease and ischemic heart disease[123].

Several studies have proved that PA decreases the blood lipids profile in individuals with T1DM[69, 74,80,93,98,101-103,110,111,124-128]. Laaksonen *et al*[125] found that endurance training improved the lipid profile in physically active T1DM men. Anaerobic circuit training also improved the lipid profile in adolescents with IDDM[96]. Similarly, regular PA improved the blood lipid profile and reduced body adiposity in children with T1DM[98]. In addition, there is a linear dose-response relationship between augmented PA and loss of abdominal fat in T1DM[91]. Regular exercise had beneficial effects on body fat content and the lipoprotein profile in subjects with T1DM by decreasing high plasma lipoprotein(a) concentrations[128]. Similarly, PA improved the lipoprotein profile in T1DM patients[102]. Increased PA in children with T1DM is related to a lower lipoprotein level[80]. Daily training for a number of months in T1DM females had a significant effect on the HDL3-C subfraction but led to minor changes in serum lipoprotein profiles[74]. Austin *et al*[127] demonstrated that the state of physical fitness was significantly correlated to lipid levels and lipoprotein(a) in adolescents with IDDM, where higher physical fitness levels decreased lipid levels. Total cholesterol (TC) levels significantly declined after an exercise intervention[124]. Consistent supervised strength training among male patients with T1DM was associated with a reduced TC level[93]. A systematic review also showed that PA reduced the TC level in children and adolescents with T1DM[97]. In addition, physical training in IDDM leads to reduced TC levels[126]. Yki-Jarvinen *et al*[75] found that a controlled physical training program in pump-treated diabetic patients increased the HDL-C to TC ratio in T1DM subjects. Postprandial walking exercise in pregnant women with T1DM was associated with significantly lower fasting plasma triglyceride levels in an intensive perinatal diabetes program[77]. Regular moderate to intense PA or sports participation improved the lipid profile in T1DM subjects[103]. Also, augmented LTPA between the ages of 50 and 70 years in the absence of active intervention was associated with improved lipid metabolism in men[101].

ENDOTHELIAL FUNCTION

Evidence shows that endothelial dysfunction is common in adolescents with T1DM[129] and it is a predictor of CVDs in these patients[130]. Studies have found that PA improves endothelial function in individuals with T1DM[110,131,132]. Anaerobic exercise training can improve endothelial function in different vascular beds in individuals with long-standing T1DM who are at substantial risk of diabetic angiopathy[131]. However, regular exercise training involving the lower extremities did not improve endothelial function in the micro- and macro-circulation of the non-exercised upper extremity in T1DM individuals[132].

INSULIN RESISTANCE

T1DM subjects are insulin resistant compared with nondiabetic subjects[133], and insulin resistance in the liver and skeletal muscle was found to be a significant characteristic in T1DM[134,135]. Youths with T1DM have adipose, hepatic, and peripheral insulin resistance[136]. This insulin resistance is an independent risk factor for the development of macro-and microvascular complications and may also contribute to the development of the disease[137]. Insulin resistance could also impact the length of the honeymoon period, diabetic control and patterns of growth during puberty, insulin requirements and BG control at any time, the birth weight of infants born to diabetic mothers, lipid metabolism, hypertension, rates of progression to insulin dependence and eventually contribute to excess mortality [138].

Insulin resistance is linked with a greater atherogenic lipoprotein cholesterol distribution in all men and women with T1DM[139]. Gender differences in insulin resistance-associated fat distribution may clarify why T1DM increases coronary calcification in women more than in men[140]. Greater insulin resistance was found in a group of premenopausal women with T1DM compared with nondiabetic subjects which was not related to abdominal adiposity, lipids, or androgens[141].

Studies have shown that PA improves insulin resistance in patients with T1DM[142,143]. For instance, aerobic exercise decreases waist circumference which is related to a tendency for raised HDL-C levels, and this may indicate a decrease in visceral fat with an improvement in insulin resistance[143]. This finding is also supported by the trial conducted by Dotzert *et al*[144] where aerobic exercise training was found to improve insulin resistance in insulin-resistant T1DM rats. The capacity of exercise to increase insulin-stimulated glucose uptake *in vivo* was decreased in subjects with insulin-resistant T1DM compared with normal individuals, and this could have been due to either separate or common defects in exercise- and insulin-stimulated pathways[142]. In β-cell transplanted recipients, endurance training may be helpful and preventive by counteracting graft dysfunction, alleviating the side effects of immunosuppressive drugs, and conserving insulin independence after islet transplantation[145].

INSULIN SENSITIVITY

T1DM adolescents have significantly reduced insulin sensitivity compared with nondiabetic adolescents [146]. Several studies have verified that PA improves insulin sensitivity in patients with T1DM[39,75,83,84,92,98,101,126,147,148]. Augmented LTPA levels were associated with raised insulin sensitivity[84,101]. Regular PA enhanced insulin sensitivity in children with T1DM[98]. Also, a controlled physical training program in pump-treated T1DM subjects improved body sensitivity to insulin[75]. Exercise training in adolescents with T1DM can lead to improved insulin sensitivity[92]. Physical training in IDDM also leads to raised peripheral insulin sensitivity[126]. Regular moderate-intensity PA can improve insulin sensitivity in T1DM individuals[39]. The findings from the trial, where exercise led to improved insulin sensitivity and responsiveness by different mechanisms in rats[149] also supports these studies.

INSULIN REQUIREMENT

PA also decreases insulin requirements in subjects with T1DM[75,84,128]. A controlled physical training program in pump-treated T1DM subjects decreased insulin requirements[75]. In addition, male patients with T1DM appeared to use less insulin when they were physically active[84]. Furthermore, regular exercise[128] and combined exercise training (aerobic and resistance) appears to lower daily insulin requirements in patients with T1DM[118]. Similarly, a study showed that exercise can improve insulin requirements in T1DM rats[150].

CARDIOVASCULAR DISEASES

Normal weight adolescents with T1DM have impaired autonomic function and augmented energy expenditure and fat oxidation compared to individuals without diabetes who have similar levels of fitness and PA[151]. T1DM adolescents had significantly reduced peak oxygen consumption (VO2peak), and peak work rate compared with nondiabetics. They also had decreased vascular reactivity, diastolic dysfunction, and left ventricular hypertrophy[146]. Maximal workload and oxygen uptake were markedly diminished in chronically hyperglycemic IDDM subjects and physiologically significant cardiopulmonary dysfunction developed in asymptomatic patients with long-standing disease[152]. Insulin resistance in T1DM may contribute to the augmented CVD burden[120]. In T1DM, heart rate variability and arterial wall stiffness are linked to each other where the autonomic nervous system could be a connection between diabetes and vascular disease[153]. In African Americans with T1DM, high

plasma interferon-inducible protein 10 was found to be an independent predictor of incident CVD[154].

Studies have confirmed that PA decreases the risk of CVDs in T1DM patients[39,80,91,104,110,112,124,127,128,143,155-159]. Increased PA in children with T1DM has a beneficial effect on the CV risk profile[80]. There is a linear dose-response relationship between augmented PA and a decrease in lipid-related CV risk factors, with a preferential rise in the HDL3-C subfraction in patients with T1DM[91]. Exercise also improves diabetic complications such as subclinical autonomic neuropathy and CVD risk in children with T1DM[124]. Higher physical fitness levels due to exercise decrease lipid levels and this, in turn, may reduce the risk of CVD[127]. In addition, regular exercise may decrease CV risk in T1DM patients by decreasing high plasma lipoprotein Lp(a) concentrations[128]. Aerobic exercise decreases waist circumference which is related to a tendency for raised HDL-C levels, and this may indicate a decrease in visceral fat with an improvement in insulin resistance which could have an influence on reducing CV risk in these patients[143]. An inverse association was found between PA and the incidence of CVD in women with T1DM[155]. Also, high frequency and high-intensity exercise may decrease the risk of CVD in individuals with T1DM[156]. Regular moderate-intensity PA can decrease the risk of CVD in T1DM individuals[39]. Furthermore, PA has the potential to delay CVD in T1DM as it reduces the risk of CVD[104,112,157]. Integrated with diet, it can also influence lipid-related CV risk factors independent of changes in insulin treatment[158]. T1DM subjects who are physically more active have a lower overall risk of CV events than their sedentary counterparts[159]. Moreover, the study showed that aerobic circuit training was found to improve cardiorespiratory endurance[96] and regular PA improved vascular health in those subjects[160].

BLOOD PRESSURE

PA also improved blood pressure (BP) in patients with T1DM[80,91,102,124,128]. A study showed that regular exercise was found to have beneficial effects on BP in these patients[128]. Increased PA in children with T1DM leads to improved diastolic BP (DBP)[80]. A linear dose-response relationship between increased regular PA and decreased BP in patients with T1DM was also found[91]. DBP and heart failure were significantly correlated with lower TC levels which were lowered by performing exercise[124]. In addition, adolescents with T1DM have been associated with reduced stroke volume during exercise[161]. T1DM adolescent girls showed decreased sympathetic activity, although this was possibly compensated by higher adrenomedullary responsiveness or sensitivity, and did not affect their heart rate adaptation to exercise[162].

WELL-BEING

Studies have also found that PA improves the well-being of individuals with T1DM[39,69,97,98,118]. A systematic review showed that PA improved the well-being and psychological health of children and adolescents with T1DM[97]. Regular PA enhanced psychosocial well-being in children with T1DM[98]. A randomized trial demonstrated that combined exercise training (aerobic and resistance) appeared to lead to better well-being in adolescents with T1DM[118]. Also, regular moderate-intensity PA can improve the psychological well-being of these individuals[39]. Furthermore, Brazeau *et al*[163] demonstrated an association between greater PA and a better body mass index, body composition, and more favorable health status in individuals with T1DM similar to individuals without diabetes.

BODY'S DEFENSE SYSTEMS

T1DM individuals have higher levels of free radicals and may, as a result, be at augmented risk of developing complications related to T1DM[164]. PA has been found to protect against protein denaturation[165]. Aerobic training improves oxidative stress in individuals with diabetes[110]. Acute exercise is an immune system adjuvant that improves defense activity and metabolic health. In addition, there is a clear inverse relationship between moderate exercise training and illness risk[166]. Farinha *et al*[9] demonstrated that exercise training improves the body's defense systems and metabolic health in T1DM patients and induces numerous benefits by decreasing inflammation and improving antioxidant defenses.

MORTALITY

Mortality rates in the past decade continue to be much larger in individuals with T1DM than in those without diabetes despite advances in inpatient care[167]. The risk of death rises with less favorable

glycemic status and impaired carbohydrate metabolism contributes to mortality from any cause[168]. The mortality rate due to ischemic cardiac disease is greater in T1DM patients compared with the general population[169]. In these patients, the presence of metabolic syndrome is frequent, and it is linked with an augmented incidence of chronic complications and mortality[170]. Macrovascular and microvascular disease are the main causes of mortality in T1DM[130]. In addition, physical inactivity has been found to contribute to a substantial number of deaths in those with DM[171]. A significantly increased mortality risk due to diabetes was associated with decreased health-related quality of life in subjects who reported no LTPA[172].

Many studies have shown that PA decreases mortality in subjects with T1DM[93,143,155,172-175]. Supervised strength training in men with T1DM was related with no morbidity[93]. An inverse association was found between PA and all-cause mortality in both genders with T1DM[155]. Participating in LTPA may be linked with improved survival in patients with diabetes[172]. Furthermore, exercise is associated with a lower risk of premature all-cause mortality such as CVD and chronic kidney disease in patients with T1DM[173], and PA has been found to decrease CV mortality in these subjects[143,174]. Moreover, PA offers a beneficial effect in terms of long life in IDDM patients [175]. In African Americans with T1DM, low plasma stromal-derived factor-1 was found to be an independent predictor of mortality[154].

THE BARRIERS TO PHYSICAL ACTIVITY PRACTICE IN T1DM PATIENTS

Even though several literature reports have supported the utilization of PA for individuals with T1DM, most of the patients did not engage in regular exercise due to various obstacles. For instance, the fear of exercise-induced hypoglycemia is the strongest barrier to regular PA in adults with T1DM[39,176-180]. Although technological advances have permitted exercisers with diabetes to progress toward more successful management of their BG levels during various types of PA, technology is still far from fully avoiding the fear of hypoglycemia in T1DM subjects[176]. Glucoregulatory failure may cause hypoglycemia in IDDM individuals during and after exercise. This could be due to hypoglycemic episodes which blunt the glucoregulatory response to subsequent exercise while exercise blunts the glucoregulatory response to subsequent insulin excess[181]. Even though regular PA was found to improve glycemic control, its frequency is a major factor affecting the control of glycemia without raising the risk of severe hypoglycemia in pediatric patients with T1DM[94]. This may be supported by a study, where low levels of LTPA were associated with poor glycemic control in T1DM women[84].

Adolescents with T1DM who participate in moderate-intensity exercise in the afternoon have augmented glucose needs at the time of and shortly after the completion of exercise. In addition, the reduced counter-regulatory responses to hypoglycemia post-exercise may lead to a higher risk of hypoglycemia overnight[182]. Antecedent hypoglycemia induces acute counter-regulatory failure both during subsequent hypoglycemia and moderate exercise in T1DM. This acute state of counter-regulatory impairment may be one cause of exercise-associated hypoglycemia in these individuals[183]. Anaerobic exercise usually causes BG concentration to reduce quickly, whereas anaerobic exercise may cause it to increase, making glycemic control challenging for patients with T1DM[184]. Prolonged exercise could lead to hypoglycemia even in normal male individuals[185]. Even though the risk of exercise-induced hypoglycemia is a great challenge for these patients, the glycemic response to exercise depends upon several factors concerning the patient him/herself such as therapy, glycemic control, training level, and the characteristics of the exercise performed[186].

Also, evidence shows that there are sex-related differences in exercise responses that might affect BG levels during exercise in patients with T1D[187,188]. Marked sexual dimorphism occurs in the pattern of counter-regulatory responses to moderate, prolonged euglycemic exercise in subjects with T1DM. Despite decreased plasma levels of epinephrine, norepinephrine, and growth hormone, T1DM women have a higher lipolytic response, which probably reflects greater tissue sensitivity to one of these hormones during exercise[188]. When compared with men, women with T1DM are more resistant to the blunting effects of antecedent hypoglycemia on neuroendocrine and metabolic responses to subsequent moderate exercise[189].

A study showed that patients with T1DM have a variable glycemic response to prolonged aerobic exercise, and this variability is partially explained by their pre-exercise BG levels[190]. High-intensity interval training (HIIT) has been found to improve anaerobic capacity without a detrimental decrease in BG in these patients[191]. However, another study showed that HIIT in fasting individuals with T1D produces a large and consistent hyperglycemic response instantly post-exercise[192]. Also, Fahey *et al* [193] demonstrated that a sprint as short as 10 s can raise plasma glucose levels in nondiabetic and T1DM subjects, with this increase resulting from a transient decrease in glucose rate of disappearance (Rd) rather than from a disproportionate rise in glucose rate of appearance (Ra) relative to glucose Rd as reported with intense aerobic exercise. Furthermore, other identified barriers were lack of time and work-related factors, access to facilities, lack of motivation, embarrassment and body image, weather, and having low levels of knowledge about managing diabetes and its complications in relation to exercise as the main barriers to perform exercise[194].

THE STRATEGY TO CONTROL PHYSICAL ACTIVITY INDUCED HYPOGLYCEMIA IN T1DM

The effective management of T1DM desires a multidisciplinary combined method to develop individualized programs, attention to all factors that may influence the result, and the expectations of those with TIDM should be paramount in the strategy adopted by the diabetes care team[195]. It is essential to know that both hypoglycemia and hyperglycemia can arise during exercise; however, strategies are available to deal with these challenges[64]. In T1DM, due to the potential risk of hypoglycemia, the patients must be carefully educated about the consequences of PA on their BG levels and the modifications of diet and insulin therapy before starting exercise sessions[196].

It is supportive for subjects to monitor their BG levels before, during, and after exercise, to avoid T1DM complications and to identify when changes in insulin or food intake are essential. In particular, individuals who experience late or nocturnal hypoglycemia should have a snack after exercise and/or before going to sleep[197]. It is suggested that the personalized exercise carbohydrate requirement estimation system can be used for the management of exercise-related glycemic imbalances in T1DM [198]. In individuals with T1DM being treated with intensive insulin therapy containing the basal-bolus (NPH-human regular) insulin regimen, walking after meals improves glycemic control[199]. Also, performing resistance exercise before aerobic exercise improves glycemic stability throughout exercise and decreases the duration and severity of post-exercise hypoglycemia in subjects with T1DM[200]. Performing a morning resistance exercise session after an overnight fast and omission of pre-exercise rapid-acting insulin does not induce acute post-exercise hypoglycemia or increase the marker of muscle damage in T1DM patients[201]. In addition, morning exercise reduces the risk of late-onset hypoglycemia compared with afternoon exercise and improves BG control the following day[109]. Eating low glycemic index food with a decreased rapid-acting insulin dose following evening exercise avoids postprandial hyperglycemia and inflammation and provides hypoglycemia protection for nearly 8 h post-exercise[202]. Ingested carbohydrates before moderate-intensity exercise with added repeated sprints is not significantly detrimental to glycemic management in overnight fasted people with T1DM under basal insulin conditions[203]. Also, a qualitative study among athletes with T1DM showed that peer mentoring and mobile apps could potentially support the management of glycemic control in athletes[204]. In athletes with T1DM, while the reductions in glucose level during continuous moderate-intensity exercise and combined (continuous moderate-intensity and intermittent high-intensity) exercise are analogous, the latter form of exercise protects against nocturnal hypoglycemia which indicates that continuous moderate-intensity exercise is linked with a raised risk of nocturnal hypoglycemia in these patients[205].

Furthermore, a larger insulin basal rate decrease and supplemental carbohydrates during exercise may be essential to avoid hypoglycemia[206]. In addition, a combination of ideal glycemic control, empirical adjustments of insulin administration at the time of exercise, and ingestion of carbohydrate supplements tailored to the type, intensity, and duration of an exercise also help to prevent hypoglycemia[207]. Exercise has a role in insulin pump therapy and improves metabolic control in patients with T1DM[208]. A reduction in the basal rate during fasting exercise in continuous subcutaneous insulin infusion-treated individuals seems to be a reasonable step in the maintenance of near-normoglycemia in individuals in whom this occurs[209].

However, reducing the basal insulin infusion rate by 80% up to 40 min pre-exercise onset was found to be insufficient to decrease exercise-induced hypoglycemia[210]. In children, even discontinuing basal insulin during exercise is an effective approach for reducing hypoglycemia in children with T1DM, but the risk of hyperglycemia is increased[211]. Another approach is short-time hypoxia together with graded exercise, which increases cardiorespiratory adaptation to exercise and permits more effective control of glucose homeostasis in T1DM[88]. Combining exercise with hypoxia may permit more effective short-term glycemic control in these patients[212]. Besides, home-HIT appears to provide a strategy to decrease the fear of hypoglycemia, while simultaneously eliminating other identified barriers in individuals with T1DM from performing exercise such as being time-efficient, no travel time or costs related to gym memberships, and providing them with the chance to exercise in their chosen environment, decreasing embarrassment experienced by some when exercising in public[213].

Technology such as continuous glucose monitoring (CGM) is a strategy to control hypoglycemia during exercise in T1DM, and it allows individuals to see the trends in glycemic fluctuations when exercising and in the subsequent night to deal pre-emptively with hypoglycemic risks and treat hypoglycemic episodes in a timely way[214]. Using CGM during exercise may avoid exercise-induced hypoglycemia, but usual BG control should be carried out during intensive exercise[215]. High-intensity exercise leads to delayed nocturnal hypoglycemia and CGM is a useful approach in T1DM subjects who undergo an exercise program[216].

Using CGM trends and carbohydrate intake based on standard exercise carbohydrate intake guidelines to facilitate exercise in children with T1DM is effective as both were found to minimize hypoglycemia and maintained euglycemia during exercise in young children with T1DM[217]. In addition to this, real-time continuous glucose monitoring (RT-CGM) can be recommended as an extra tool that offers T1DM adolescents a rapid reaction to decrease glycemic variability within a short time [218]. Besides, RT-CGM with a carbohydrate intake algorithm may avoid hypoglycemia and maintain euglycemia during exercise, mainly if the subject consumes carbohydrates when trend arrows alert

them to a drop in glycemia[219].

Furthermore, closed-loop insulin delivery also offers an effective means to decrease the risk of nocturnal hypoglycemia while increasing the percentage of time spent in the target range, irrespective of activity level during mid-afternoon. This could benefit these patients even if it is limited to the overnight period[220]. The hybrid closed-loop systems are another approach that help to avoid hypoglycemia, relying on accurate carbohydrate ratios and carbohydrate counting, and the algorithm that was tested against moderate exercise and an over-reading glucose sensor performed well in terms of hypoglycemia prevention[221]. Moreover, a study demonstrated that the heart rate–enhanced artificial pancreas system improved protection against hypoglycemia during exercise in T1DM[222]. Several studies have shown different strategies that could be integrated with exercise as a means of glycemic control in individuals with T1DM (Table 1)[223-240].

PHYSICAL ACTIVITY RECOMMENDATIONS FOR T1DM PATIENTS

It is essential to balance the risks of insulin-induced hypoglycemia with the risks related to poorly controlled diabetes and poor physical fitness in individuals with T1DM[241]. Considering the risk-benefit ratio, several studies have recommended PA in patients with T1DM[9,71,79,92,97,113,124,158,191,207,214,231,242-248]. Regular PA should be a routine aim in these subjects, for various health and fitness reasons. However, considerable challenges remain for these patients, and their healthcare team, in the management of exercise and sports[244]. Exercise is highly recommended for patients with T1DM as it has several beneficial health effects, with the prevention of long-term cardiovascular complications being dominant[207,214].

With regard to exercise, regular moderate-to-vigorous exercise should become a central part of the management of subjects with T1DM, in the absence of contraindications and accompanied by all desirable educational support for optimal diabetes management[245]. Children with IDDM can be engaged in regular vigorous PA (with minimal risks)[71] and regular exercise[124]. A combined exercise of strength training (ST) and HIIT for at least 2 mo, 3 times per week, will provide many health benefits for T1DM subjects[9].

Another study showed that HIIT sessions reduced glycemia to a greater degree than ST or ST+HIIT sessions over 10 wk in real-life situations. Because of this, T1DM individuals who develop severe exercise-associated hypoglycemia and/or present pre-exercise capillary glucose levels close to 5.5 mmol/L are recommended to carry out ST or HIIT after ST as the preferred option[247]. Resistance exercise was also found to have several benefits and should be recommended as a significant activity for health and well-being in these individuals, although caution with regard to BG levels will always be essential while performing resistance exercise[231]. However, HIIT may be the chosen training approach for some individuals with T1DM as it has been found to improve anaerobic capacity without a detrimental decrease in BG in these patients[191]. For T1DM patients using ultra-long-acting insulin, both aerobic high-intensity interval exercise and moderate continuous exercise can be safely performed [67].

In addition, high-intensity exercise does not raise the risk of early post-exercise hypoglycemia in patients with T1DM[249]. High-intensity bouts linked with high-intensity exercise result in a more rapid and higher increase in endogenous glucose production during exercise than moderate-intensity exercise alone. During early recovery from exercise, glucose use reduces following high-intensity exercise, while leftovers raised after moderate-intensity exercise despite the performance of more whole work[250]. In pediatric patients with T1DM, the frequency of regular PA was the main factor that affected the control of BG without raising the risk of severe hypoglycemia and this is why it is recommended in these patients[94].

Moreover, for subjects with T1DM, the emphasis must be on adjusting the therapeutic regimen to allow safe participation in all forms of PA consistent with an individual's desires and goals. Eventually, all subjects with diabetes should have the chance to benefit from the valuable effects of PA[248]. Daily PA should be recommended in these patients as part of their management[113] and could be used as an adjunct in glycemic control[92]. Diet and PA can influence glycemic control in IDDM independent of changes in insulin treatment[158].

Besides exogenous insulin therapy and CGM, exercise is recommended in adults with T1DM to improve the entire health of individuals[79]. However, to perform regular PA, the patient and those who supervise them should be aware of disease-specific recommendations and contraindications[242]. Evidence recommends that even individuals with ketonemia may engage in intensive physical training, provided this is part of a program including adequate insulin dosage, dietary advice, and close supervision with multiple daily BG measurements[246]. Evidence suggests that it is significant to consider the needs of the wider support network, as well as the child's or adolescent's concerns and preferences, during the development of new or existing strategies and programs to promote PA in children and adolescents with T1DM[251]. Finally, during PA, the parameters such as supervision, duration, frequency of sessions, protocols with mixed PA may positively affect the metabolic outcome of patients with T1DM[97].

Table 1 Summary of studies on exercise intervention in type 1 diabetes mellitus patients to improve glycemic control

Ref.	Year	Intervention	Findings
Sonnenberg *et al*[223]	1990	CSII during exercise	Hypoglycemia could only be avoided when the premeal insulin bolus was decreased by 50% and discontinuation of the basal insulin infusion during exercise
Rabasa-Lhoret *et al*[224]	2001	Premeal insulin dose reductions forpost-prandial exercises	Minimized risk of hypoglycemia during postprandial exercises of different intensities and different durations by a suitable decrease in premeal insulin lispro
Dubé *et al*[225]	2005	Glucose supplement during exercise in subjects using N-lispro	For 60 min of late post-prandial exercise followed by 60 min of recovery, an estimated 40 g of a liquid glucose supplement, ingested 15 min before exercise was good for BG control
Diabetes Research in Children Network (DirecNet) Study Group *et al*[211]	2006	Suspension of basal insulin during exercise	Basal insulin suspension decreases hypoglycemia from 43% to 16% in individuals, but hyperglycemia 45 min after exercise was more frequent
Bussau *et al*[226]	2006	Ten-second sprint after moderate-intensity exercise	This avoided early post-moderate intensity exercise hypoglycemia
Bussau *et al*[227]	2007	Ten-second sprint before moderate-intensity exercise	Prevented hypoglycemia during early recovery from moderate-intensity exercise
West *et al*[228]	2010	Reductions in pre-exercise rapid-acting insulin by 75%, 50%, or 25%	A 75% reduction in pre-exercise insulin resulted in the greatest preservation of BG, and a decreased dietary intake, for 24 h after running
Taplin *et al*[229]	2010	20% reduction of basal rate overnight	Was safe and effective in preventing nocturnal hypoglycemia
		2.5 mg bedtime dose of oral terbutaline	Effective at avoiding hypoglycemia, but linked with hyperglycemia
Riddell *et al*[219]	2011	RT-CGM and carbohydrate intake algorithm (8-20 g), depending on the concentration of glucose at the time of RT-CGM alert and rates of change in glycemia	The coupled carbohydrate intake algorithm with RT-CGM avoided hypoglycemia and maintained euglycemia during exercise
Garg *et al*[230]	2012	An automatic suspension of insulin delivery when BG ≤ 70 mg/dL during or after exercise	This significantly decreased the duration and severity of induced hypoglycemia without causing rebound hyperglycemia
Yardley *et al*[200]	2012	Resistance exercise before aerobic exercise	Performing resistance first improved glycemic stability throughout the exercise and decreased the duration and severity of post-exercise hypoglycemia
Yardley *et al*[231]	2013	Resistance *vs* aerobic exercise	Resistance caused a less initial decline in BG but prolonged decreases in post-exercise glycemia than aerobic exercise
Campbell *et al*[232]	2013	Pre- and post-exercise rapid-acting insulin reductions	25% pre-exercise and 50% post-exercise rapid-acting insulin dose preserved glycemia and protected patients against early-onset hypoglycemia (8 h)
Schiavon *et al*[233]	2013	In silico optimization of basal insulin infusion rate during exercise	A decrease in basal insulin by 50% starting 90 min before exercise and by 30% during exercise is safe and effective for glucose control
Danne *et al*[234]	2014	PLGM (suspension of insulin delivery based on predicted sensor glucose values)	PLGM may decrease the severity of hypoglycemia above that already established for algorithms that use a threshold-based suspension
Campbell *et al*[235]	2015	Combined basal-bolus insulin dose reduction and carbohydrate feeding strategy following exercise	Reducing basal-bolus insulin by 20% (80%) protected from nocturnal hypoglycemia for 24 h post-exercise
Cherubini *et al*[236]	2019	PLGM system during exercise	Effective for avoiding hypoglycemia during and after exercise, regardless of the thresholds of PLGM used
Moser *et al*[237]	2019	Oral administration of carbohydrates during moderate-intensity exercise	Pre-exercise BG levels determine the amount of orally administered carbohydrates during exercise to maintain euglycemia
Zaharieva *et al*[238]	2019	Basal rate reductions set 90 min pre-exercise *vs* pump suspension at exercise onset	50%-80% Basal rate reductions set 90 min pre-exercise improved BG control and reduced hypoglycemia risk during exercise better than pump suspension at exercise onset
Moser *et al*[239]	2019	Reduction in insulin degludec dose (75% IDeg dose *vs* 100% IDeg dose)	Reducing the usual IDeg dose by 25% led to more time spent in euglycemia with small effects on time spent in hypo- and hyperglycemia

Zaharieva *et al*[240]	2020	Insulin pump connected (pump on) *vs* pump disconnected (pump off) during high-intensity exercise	No significant differences in BG concentrations during 40 min of intermittent high-intensity exercise

CSII: Continuous subcutaneous insulin infusion; BG: Blood glucose; RT-CGM: Real-time continuous glucose monitoring; PLGM: Predictive low glucose management.

CONCLUSION

As T1DM is rising globally, PA is recommended as evidence shows that PA can control the burden of disease. The benefit of PA in T1DM is a significant component in its management. It is recognized as having beneficial effects and is key to a healthy lifestyle as well as the management of T1DM. PA can be considered an efficient and inexpensive non-pharmacologic tool for the management of T1DM in addition to insulin therapy. It has significant health benefits such as improves BG control, physical fitness, endothelial function, insulin sensitivity, well-being, and the body defense system. In addition, it reduces the blood lipid profile, insulin resistance, CVDs, insulin requirements, BP, and mortality associated with T1DM.

Overall, ideal glucose control is of principal significance in T1DM including during PA. Previously, it is challenging to prevent the hazards associated with PA in these individuals. Hypoglycemia and fear of hypoglycemia are the greatest challenges in T1DM individuals engaging in PA and can limit suitable glycemic control in these patients. However, a better understanding of energy metabolism and homeostasis has made it possible for individuals with diabetes to take part in exercise. In addition, several strategies have been identified to make PA more suitable for T1DM individuals. In particular, improvements in glucose monitoring technology and the availability of other interventional approaches during PA have further contributed to the feasibility of exercise programs for these subjects. There are also strategies for preventing exercise-induced hypoglycemia during and after exercise.

The present review may help health professionals to encourage PA as part of the management of individuals with T1DM. It is also recommended that PA can be performed carefully with reference to diabetes guidelines. Therefore, health professionals in clinical practice should inform and encourage patients with T1DM to manage exercise-induced hypoglycemia. Furthermore, in-depth knowledge of factors such as gender, therapy, glycemic control, training level and characteristics of the exercise performed will allow the development of individualized strategies to minimize the risk of hypoglycemia as the glycemic response to exercise depends upon these factors.

Moreover, health professionals should distinguish between hyperglycemia induced by HIIT and the concern of hypoglycemia-related to less intense forms of exercise during patient counseling for T1DM. Also, this should be clearly set out in practice guidelines. Patients and health professionals should be aware of the degree and duration of post-HIIT hyperglycemia and the potential benefit of an insulin correction bolus. Support for patients on how better to control their BG after exercise could encourage these patients to be less fearful of exercise-induced hypoglycemia and participate in regular PA. Clear-cut quantitative approaches to prevent exercise-induced hypoglycemia are desired to allow patients to engage in regular PA and enjoy its beneficial aspects. It is essential to consider the potential alterations in exercise responses that may occur in T1DM and not to judge this activity as harmful, but these alterations can reduce its full beneficial health effects. Lastly, the present review also provides suggestions for the future direction of research on the types of exercise, duration, and intensity recommended for T1DM patients, considering the individual's factors that could be detrimental to the response that occurs during and after exercise.

FOOTNOTES

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REVIEW

Maternal low protein diet and fetal programming of lean type 2 diabetes

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Abstract

Maternal nutrition is found to be the key factor that determines fetal health *in utero* and metabolic health during adulthood. Metabolic diseases have been primarily attributed to impaired maternal nutrition during pregnancy, and impaired nutrition has been an immense issue across the globe. In recent years, type 2 diabetes (T2D) has reached epidemic proportion and is a severe public health problem in many countries. Although plenty of research has already been conducted to tackle T2D which is associated with obesity, little is known regarding the etiology and pathophysiology of lean T2D, a variant of T2D. Recent studies have focused on the effects of epigenetic variation on the contribution of *in utero* origins of lean T2D, although other mechanisms might also contribute to the pathology. Observational studies in humans and experiments in animals strongly suggest an association between maternal low protein diet and lean T2D phenotype. In addition, clear sex-specific disease prevalence was observed in different studies. Consequently, more research is essential for the understanding of the etiology and pathophysiology of lean T2D, which might help to develop better disease prevention and treatment strategies. This review examines the role of protein insufficiency in the maternal diet as the central driver of the developmental programming of lean T2D.

Key Words: Type 2 diabetes; Maternal low protein diet; Fetal programming; Lean diabetes; Developmental origin of health and disease

Core Tip: This is to review the role of maternal low protein diet and its metabolic impact on the offspring leading to lean type 2 diabetes.

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INTRODUCTION

Type 2 diabetes (T2D) is a metabolic disease, which is rapidly increasing among the human population both in developed as well as in developing countries. Diabetes is divided into four major categories: type1, type2, gestational, and other specific diabetes mellitus[1]. As per the national diabetes statistics reports in 2020, 10.5 % (34.2 million) of the United States population are diagnosed with some form of diabetes and 34.5% (88 million) of adults have pre-diabetes. T2D constitutes 90%-95% of all diabetes cases in the United States[2]. The hallmarks of T2D are insulin resistance and insulin deficiency. In most cases of T2D, the etiology of insulin resistance and insulin deficiency can be traced back to obesity and lifestyle aspects.

Scientists from all over the world have spent a great deal of time and effort to understand the causes and consequences of obesity-induced T2D. However, the number of non-obese/lean T2D cases has also dramatically increased globally and especially in Asia and other developing countries. Recent estimates show that 10%-20% of T2D patients are not obese[3]. Although the etiology is not clearly understood, lean T2D is clustered under the umbrella of T2D for patient care. Interestingly, clues from various studies indicate that lean T2D is often observed in the populations where the fetus is exposed to malnutrition during the intrauterine period or early childhood[3-7]. Adequate birth weight and size of the newborn are often considered as indicators of appropriate fetal growth rate and optimal *in utero* environment[8-10]. In contrast, deprivation of nutrition during fetal development is often marked by low birth weight and it is linked with adult-onset of metabolic diseases such as T2D[11].

Historically, observational studies from the different parts of the world firmly indicate that the individuals exposed to *in utero* malnutrition due to famine were more prone to the hyperglycemic condition in adult life compared to those who are not born during a famine[12]. For instance, the cohort exposed to Dutch famine (1944-45) *in utero* were more prone to glucose intolerance than the non-exposure cohort[13]. Similarly, data associated with the Ukrainian famine of 1932–33 have exhibited a higher incidence of T2D among the people born in the famine-affected region than in the regions where no famine was reported[14]. The link between Australian famines and T2D was studied and analysis showed a positive correlation with three years of famine and an increased number of T2D among those who were born during the famine years[12]. Further, Li *et al*[15] reported that people who were exposed to the Chinese famine in the fetal stage during 1959-1961 were more prone to hyperglycemia and T2D in their adult life, compared to those who were born after this period.

Recent studies indicate that adverse *in utero* nutrition could cause lean T2D later in life among certain ethnic and socio-economical groups, and people with certain lifestyles. Studies from India[16] (up to 26%) and Caribbean islands[17,18] (5%) report the predominance of lean T2D population. A study on American minorities showed that 13% of T2D patients are lean[4,16] with a fivefold higher incidence in Asians[4]. These observations clearly show that not all diabetics are obese and obesity does not necessarily cause T2D[3,16,19]. With > 42 million Americans experiencing food insecurity, it is a major problem even in the United States especially among the economically disadvantaged[20]. WHO estimates that 1.1 million children had ≤ 2 standard deviations for weight for height ratio (an index of protein-energy malnutrition) in the United States[21]. A recent German study shows that 38% of pregnant women did not consume enough protein[22]. With vegetarian and vegan diets gaining popularity worldwide, low protein intake is more prevalent as these diets are often low in protein[23,24]. Vegetarian mothers consume low protein diet[25,26] and give birth to children with lower birth weights, thus making them susceptible to T2D[26-28].

This atypical diabetic phenotype is known by various names such as Jamaica type diabetes, metabolically obese normal weight (MONW) diabetes, malnutrition-related diabetes mellitus, phasic insulin-dependent diabetes, tropical diabetes, mixed onset type diabetes, J, Z, M or type 3 diabetes, and ketosis resistant growth onset type diabetes[3,17,18,29-33]. This concept of MONW individuals was first proposed in 1981[29] with subsequent validations in animal and human studies[34-37] and the existence of lean T2D has been observed for decades but the etiology and pathophysiology of lean T2D are poorly understood.

The most accepted and validated hypothesis that explains the link between early nutrition and metabolic diseases in adulthood was proposed by David Barker is called, 'thrifty phenotype hypothesis'

[11,38]. This hypothesis explains how impaired *in utero* nutrition availability results in compromised fetal growth and programs subcellular and metabolic effects in the developing fetus. Further, the hypothesis suggests that the metabolic fate of an individual predisposed to T2D is decided at the early developmental stage and thus attempts to explain why a sub-population of individuals born with low birth weight are more prone to lean T2D compare to normal birth weight individuals[11]. Various subsequent studies have confirmed the reproducibility and epidemiological evidence for the 'thrifty phenotype hypothesis'[39].

In addition, studies based on this hypothesis showed the importance of a sufficient amount of protein in the maternal diet for the development of the fetus and the risk of diseases in adulthood[40]. Although overall well-balanced nutrition is essential for a developing fetus and a healthy offspring, the role of protein is vital in the developmental programming paradigm[41]. A low protein diet is well known to cause various programming effects leading to metabolic disorders in adulthood. Low protein or vegetarian diets are consumed due to various reasons such as poverty, famine, lack of availability, cultural, religious, or moral reasons, personal preference, *etc*. Although these are common in the developing world, the recent popularity of vegetarian and vegan diets in the developed world is also an important paradigm to be considered. The emerging popularity of vegetarian and vegan diets among the maternal population might compromise growing fetuses, as the amount and bio-availability of proteins are found to be inadequate from plant sources[23,42]. A low protein diet during gestation is often connected with compromised renal function and impaired glucose metabolism[7]. However, the mechanistic basis and the exact patient phenotype of the maternal low protein associated with lean T2D are not well understood. Therefore, a clear understanding of the epidemiological and clinical features of lean T2D is essential to the prevention or treatment strategies.

PATHOPHYSIOLOGY OF LEAN T2D

T2D is a complex metabolic disease with a spectrum of presentations. It is therefore essential to understand the pathophysiology of the disease to offer appropriate prevention and treatment strategies. The pathophysiology of lean T2D is not well defined, although we and others have considered them as a separate subset of T2D[3,43,44] body mass index (BMI) is widely used as a tool to classify T2D patients. Patients with a BMI greater than 25 are considered to have obese T2D[45]. In contrast, the majority of lean T2D patients have a BMI of less than 25 but they have several metabolic characteristics associated with obesity[3]. Observational studies in humans and experiments in rodents suggested that the various environmental and genetic factors could contribute towards the lean T2D phenotype[3,46]. Poor *in utero* nutrition during fetal development is considered to be the main driver of lean T2D onset[44,46]. We have shown using a novel rat model that the maternal low protein diet is one of the critical causes of the lean T2D phenotype[43].

Although the genetic factors may vary among the different populations, genetic predisposition to fragile beta cells was found to be common in lean T2D patients. The rapid beta-cell apoptosis is the major pathophysiological characteristic of lean T2D compared to elevated insulin resistance in obese T2D[47]. Another interesting aspect that is noticed in this population is the prevalence of truncal obesity. Insulin sensitivity and insulin response are varied among the different ethnic groups (African, Caucasian, and East Asian), and East Asians have more vulnerable beta cells which make them more prone to T2D[48]. Several studies on the South East Asian population have shown that lean T2D patients have central obesity or elevated visceral fat deposits[49]. Even though lean T2D patients have lesser hyperglycemic values, their hemoglobin A1c levels are significantly higher than their obese counterparts[5,44]. Further, the onset of lean T2D is reported at an early age than the obesity-associated T2D[3]. Lean T2D patients showed a significantly lower incidence of hypertension and cardiovascular diseases compare to obese T2D patients but are more susceptible to peripheral neuropathy[3,5]. Apart from environmental and hereditary factors, socioeconomic background is found to be an important aspect of lean T2D prevalence[10,50]. Several studies have reported an inverse relationship between T2D and socioeconomic status[51]. The National Health and Nutrition Examination Survey data indicated this relationship of poverty and higher incidence of T2D among African and Mexican origins in the United States[52]. The Chicago cohort study further showed the prevalence of lean T2D among this minority community[4].

The two crucial characteristic features of developing countries, which make them more vulnerable to lean T2D, are a rapid shift in lifestyle and impaired nutrition. Studies from India have reported the escalating number of lean T2D cases across the country, especially, the urban population of India[53-55]. Similarly, Alemu and the group reported the increased number of lean T2D like cases in the urban population of sub-Saharan Africa[56].

GESTATIONAL LOW PROTEIN PROGRAMMING AND SEX DIFFERENCES IN LEAN T2D

Sex differences in fetal development can be observed as early as the pre-implantation phase[57]. There are major *in utero* differences between the sexes in growth and metabolic parameters leading to a faster fetal growth in males when compared to females. These differences are attributed to the genes expressed by sex-chromosomes and the actions of sex hormones[57-59]. In addition, differences in the incidence of T2D can also be attributed to the differences in the leptin and insulin sensitivity between sexes[59,60]. These metabolic hormones are influenced by the *in utero* nutritional environment[61]. Many studies have found a link between T2D and maternal low protein diet[43,59,62,63]. Further, as the nutritional environment often regulates the epigenetic machinery, any change *in utero* nutritional status may cause permanent alterations in the fetal gene expressions[64].

Our research using a lean T2D rat model indicates a clear sex difference in glucose homeostasis with females developing glucose intolerance earlier in life with faster disease progression than males[43,65]. These animals also showed differential regulation of gluconeogenesis and glycogenolysis as a result of gene expression changes in key genes involved in glucose metabolism[65-68]. Sex difference in hepatic genes associated with glucose homeostasis such as phosphoenolpyruvate carboxykinase (PEPCK) and 11β-hydroxysteroid dehydrogenase type 1 were observed even in low protein programmed fetuses[69]. Similarly, low protein programmed mice offspring were found to have lower birth weight with more glucose intolerant than the controls[70]. In this study, maternal low protein diet activated the visceral adipose tissue neuropeptide Y-Y2 receptor system in female offspring but not in male offspring, which increased abdominal adiposity and insulin resistance in female offspring[70]. This study indicates the importance of neuropeptide Y-Y2 receptor as a potential sex-specific marker and mediator of metabolic programming[70].

In the last decade, various studies have shown the importance of mitochondrial health and its relationship with glucose homeostasis in low protein programming. Zambrano and group have found maternal low protein diet-induced insulin resistance in male Wistar rats; however, females were responsive to glucose[71]. This study, further, suggested that elevated mitochondrial dysfunction in the pancreatic islets of adult male rats might be the mechanism that leads to insulin resistance[71]. Likewise, male offspring of low protein diet-fed mothers showed higher ROS production and impaired electron transport chain function in the mitochondria of the pancreatic islets when compared to female offspring indicating mitochondrial incompetence in males could predispose them to T2D[72]. Similarly, the sex dependent fetal programming in glucose metabolism was also reported *in utero* low protein programmed piglets. In this study, hepatic gluconeogenesis in newborn male piglets was negatively affected by the maternal low protein diet during pregnancy[73]. Epigenetic changes in the promoter region of the glucose-6-phosphatase gene were sex-specific and resulted in T2D in adult male pigs[73]. In addition, maternal low protein diet diminished liver mtDNA copy number in males and altered the OXPHOS protein expression by the combined binding action of glucocorticoid receptor and methylation of on the hepatic mtDNA promoter, which effect the mtDNA replication and gene expression levels[73,74].

Studies in humans suggest greater prevalence and impact of lean T2D in males than females. Many studies have indicated that women are physiologically inclined to have better insulin sensitivity than men[75-77]. Estrogen has a protective role in insulin sensitivity and glucose homeostasis by the inhibition of Foxo1 though activation of ERα-PI3K-Akt signaling[78]. Another crucial way estrogen protects women from insulin resistance is through mitochondrial biogenesis, as testosterone reduce mitochondrial proliferation[79]. The male preponderance of lean T2D was evident from the studies conducted in India, where more than 60% of lean T2D patients were men. Although the exact causes of sex differences are not clearly understood, it is suggested that the differences observed in this study may be due to predominant male exposure to oxidative stressors such as smoking and alcoholism[3,80].

Another interesting aspect to consider is the role of folate. Folate is routinely given to pregnant women throughout the world to prevent neural tube defect. Recent studies show that excessive folate can also have negative consequences at least in certain populations, ages and ethnicity/genetic background[81-83]. One study in Indian population shows that it can lead to insulin resistance[7]. The authors primarily attribute this to the deficiency of vitamin B12 which is primarily present in animal protein. Rat studies from our group showed that folate offered some protection in low protein programmed offspring by compensatory hyperinsulinemia but make insulin resistance worse in males [84]. Although the mechanism of this sex dependent folate action in insulin resistance is not known and warrant further research, it is important to note how folate may have sex dependent effects and this may also hold a clue in the sex differences that are observed in the human population.

ANIMAL MODELS

Considering the ethical and technical limitations in conducting impaired maternal nutrition and developmental programming studies in humans, various animal models that mimics several aspects of developmental programming have been developed. Due to the shorter lifetime and availability of

genetic tools, a substantial amount of research is presently focused on developing clinically reliable rodent models of developmental programming.

To achieve a low protein diet model, the majority of studies followed a diet that has around a 50% reduction of total protein in the diet formulation[43,63,85-87]. However, most of the investigations conserved the isocaloric nature of the diet by manipulating macronutrient proportions by various lipid and carbohydrate ratios[88-93]. Although preferred protein, carbohydrate, and lipid ratio are varied among different research groups; a single research group often stick to one specific diet regimen[67,91,94-97]. The other central deviation apparent among the different low protein models is the timing and duration of the maternal diet management. Majority of the studies have started giving low protein diet from the first day of the pregnancy and continued throughout pregnancy or lactation, although some studies initiated the low protein diet before pregnancy or in some cases in a specific period of *in utero* growth[65,86,98-102]. The main aim of these refined diet manipulations is to develop a metabolically compromised adult offspring[103]. Moreover, many studies have succeeded in mirroring low birthweight and catch-up growth pattern, which is considered by many as a hallmark of the developmental origin of metabolic disease[104-108]. Pups from maternal low protein mothers weigh less compared to those from control diet-fed mothers. The differences in birth weight disappeared once the mothers were fed with a normal diet or pups were cross-fostered with control mothers. However, the weight differences were permanent, when the maternal low protein diet was continued throughout the weaning[43,100,109]. In addition, due to the variation in macronutrients ratio and the time regime of the diet, the adult metabolic phenotypes reported by various groups are also varied. Insulin resistance, obesity, cardiovascular diseases, and dyslipidemia are the major clinical disorders observed in these models[104,110-113]. A comprehensive list of different low protein programming animal models used are summarized in Table 1.

PHYSIOLOGICAL EFFECTS AND MECHANISMS

Many animal models based on a low protein diet have been successful in capturing the phenotypic characteristics of fetal programming of adult metabolic diseases. However, the exact mechanism that leads to these metabolic diseases is not well studied. The dominant hypothesis in the field of developmental programming of adult diseases attribute that the fetal epigenome play a central role. This hypothesis postulates that epigenome is reprogrammed as an adaptation in response to a low protein diet, the associated low birth weight, and the catch-up growth. A recent study in Japanese adults indicates that the reduced beta cell mass in low-birth-weight individuals is directly associated with the future development of T2D[114]. Although the epigenome is prone to modification throughout the lifetime, *in utero* developmental period was found to be the most vulnerable time to be dysregulated by stressors[115].

Several studies have reported various key genes that are epigenetically modified as a result of developmental programming. For instance, the transcription factor Hnf4a was found to be epigenetically regulated during gestation, and the maternal diet-induced changes in the expression of this gene can cause T2D in adulthood[116]. Similarly, glucose transporter 4 (GLUT4) expression in skeletal is epigenetically controlled by maternal diet during early development and the impaired gene expression often resulted in peripheral insulin resistance[117]. Even though different biological mechanisms might contribute to fetal programming of lean T2D, many recent studies are indicating epigenetic changes as a potential single important driver of the fetal programming effects[118]. Low protein diet exposure during pregnancy in animals exhibited changes in methylation in promoter regions of genes involved in the glucose homeostasis pathway thereby, affecting the gene expression either directly or indirectly [119]. In recent years, many experimental studies in animals and observational studies in humans show that the epigenetic changes associated with gestational low protein are the main regulatory forces mediating the T2D phenotype[118,120]. Changes in the fetal epigenome often mirror the unique *in utero* environment of the fetus. Epigenetic changes due to gestational low protein arise through the methylation of cytosine in CpG Island present in the promoter region of particular genes, histone protein modification by acetylation, and regulation microRNAs by post-transcriptional modification. The chromatin structure and expression of a specific gene are regulated through DNA methylation in association with histone modifications[121].

A study in pigs found a significant decrease in glucocorticoid receptor binding to the glucose-6-phosphatase (G6PC) promoter which was accompanied by hypomethylation of the G6PC promoter in association with gestational low protein diet[74]. As G6PC is one of the crucial enzymes in glucose homeostasis that catalyzes gluconeogenesis and glycogenolysis, epigenetic changes in the promoter region might contribute to the onset of hyperglycemia[74]. Further, this impaired maternal diet-induced reduction of mtDNA copy number and methylation of mtDNA promoter often leads to changes in OXPHOS gene expression. This may predispose to insulin resistance in adult offspring considering the importance of hepatic mitochondrial OXPHOS activity in glucose homeostasis[73].

Similarly, using maternal low protein programmed rats, Lillycrop *et al*[8] established that the hepatic PPARα promoter and glucocorticoid receptors were hypomethylated *in utero* and these epigenetic

Table 1 Summary of key animal models used to investigate the maternal low protein associated insulin resistance and glucose intolerance

Animals	Diet regimen	Age of pups	Sex	Observations	Ref.
Sprague-Dawley rats	6% protein, -12 to 43 d	12 wk	Females	Sirt3 dysfunction in skeletal muscle	[138]
Sprague-Dawley rats	10% protein, 2 to 21 d	Newborn	Males	Increased *Igf* gene expression	[148]
Sprague-Dawley rats	8% protein, 1 to 43 d	17 wk	Males	Lower fasting insulin and HOMA	[85]
Wistar rats	6% protein, 1 to 21 d	11 wk	Females	Insulin resistance and glucose Intolerance	[103]
Wistar rats	6% protein, 1 to 43 d	3 wk	Both	Compromised β-cell structure and function	[163]
Wistar rats	7% protein, 1 to 120 d	16 wk	Females	Higher glucose tolerance and insulin responsiveness	[98]
Wistar rats	8% protein, 1 to 43 d	12 wk	Both	Impaired gluconeogenesis, glucose handling and liver structure	[141]
Wistar rats	8% protein, 1 to 43 d	11 wk	Females	Insulin resistance and glucose Intolerance	[190]
Wistar rats	8% protein, 1 to 21 d	12 wk	Males	Epigenetic regulation of Hnf4a in islets	[116]
Wistar rats	8% protein, 1 to 21 d	12 wk	Both	Altered mitochondrial function in islets	[72]
Wistar rats	8% protein, 1 to 21 d	12 wk	Both	Structural alterations and changes in glucokinase expression in liver	[141]
Wistar rats	8% protein, 1 to 21 d	Fetal Day 21.5	Both	Altered IGF axis and proliferative capacity of liver	[140]
Wistar rats	9% protein, 1 to 20 d	Fetal Day 20	Both	Defective hepatic glucose homeostasis	[69]
Wistar rats	10% protein, 1 to 21 d	4 wk	Both	Impaired hepatic gene expression	[8,122]
Wistar rats	10% protein, 1 to 43 d	15 wk	Both	Modified glucose metabolism and insulin resistance	[71]
C57BL/6J mice	9% protein, 1 to 39 d	8 wk	Both	Impaired glucose metabolism, miR-15b up-regulation	[63]
C57BL/6J mice	8% protein, 1 to 21 d	3 wk	Both	Altered PPAR signaling, insulin resistance and glucose Intolerance	[87]
C57BL/6J mice	8% protein, 1 to 19 d	Newborn	Both	Altered mitochondrial genes expression in liver and skeletal muscle	[89]
Mice	8% protein, 1 to 40 d	21 wk	Both	Increases abdominal adiposity and glucose intolerance	[70]
Pig	6% protein, -18 to 113 d	Newborn	Both	Affected mitochondrial OXPHOS and glucose-6-phosphatase in liver	[73,74]

changes were persistent in adulthood. Further studies demonstrated reduced Dnmt-1 expression and its role in epigenetic changes of glucocorticoid receptors[73,95,96,122]. Moreover, epigenetic changes in the promoter region of PEPCK were found to be the drawing force for impaired glucose homeostasis in animals[123,124]. Anandwardhan and colleagues reported a decreased number of (pro) insulin 2 gene transcripts in the pancreas of low protein *in utero* programmed rats, due to the histone modification in the promoter region of the insulin 2 gene[125]. Moreover, these epigenetic changes are potentially engaged in the trans-generational transmission of the induced phenotype[122,124,125].

Recently, Goyal and group demonstrated that the epigenetic modifications by miRNA, small non-coding RNAs consists of 20–22 nucleotides, is one of the molecular mechanisms of maternal low protein-induced T2D[119]. Results from maternal low protein programmed mice found reduced beta-cell mass and insulin levels in the pancreatic islets of the programmed offspring due to the increased expression of miR-15b. As the activities of cyclins are negatively regulated by the presence of miR-15b, the up-regulation of this miRNA may inhibit pancreatic beta-cell proliferation, consequently, stem to T2D phenotype[63]. A microarray study also demonstrated elevated expression of miR-615, miR-124, miR-376b, and decreased expression of miR-708 and miR-879 in maternal low protein programmed mice, which were associated with degenerated metabolic health of the offspring from the weaning age [126].

Apart from the epigenetic changes, maternal malnutrition is the major reason for low birth weight in newborns. Children who are small for gestation age and showed catch-up growth during the early age of development appeared to be more insulin resistant compared with normal-weight children[127]. Moreover, several studies have shown epigenetic changes due to gestational diet-induced fetal programming adult diseases in these offspring[108,128,129].

Even though little is known about the mechanism of programming, the secondary effects of fetal programming and their mechanisms are well studied. For example, various organ systems that play vital roles in the metabolism, and how they are affected by the developmental programming of T2D are well characterized. *In utero* low protein exposure causes long-lasting structural and functional changes in metabolically active organs includes skeletal muscle, liver, pancreas, gonads, and brain.

The *in utero* environment is crucial in the development of skeletal muscles, and the muscles growth is determined by the number, size, and type of muscle fibers formed during fetal development[130,131]. Maternal undernutrition affects the quality and quantity of skeletal muscles and stem cell activity[132-134]. A maternal low protein diet during gestation affects the normal proliferation and differentiation of bone marrow stem cells and satellite cell function[134,135]. Therefore, imperfections of skeletal muscles development during fetal development are often deleterious to normal muscle functions in adulthood [133]. Studies using maternal low protein diet-based animal models have reported lower expression of GLUT4 and mitochondrial dysfunction in skeletal muscles of low protein offspring[66,67,136-138]. As skeletal muscle functions as one of the main sites for peripheral glucose disposal, functional or structural changes of the myofibrils leads to insulin resistance and glucose intolerance[139].

Similarly, low protein-induced developmental programming caused functional and structural changes in the liver[140,141]. The expression of genes associated with oxidative phosphorylation and glucose metabolism were altered in the liver. Further, *in utero* low protein exposed rat fetuses showed the altered structure of the liver with decreased proliferation of hepatocytes[142-146]. These animals also had altered hepatic lipid metabolism and hepatic desaturase activities, which may account for fetal growth retardation and insulin resistance[94,147]. In addition, a maternal low protein diet also induces epigenetic changes in methyltransferase machinery resulting in altered epigenetic regulation in the liver [148]. Although further studies are warranted, it is clear from the existing studies that developmental programming induced by a low protein diet affects hepatic structure and function and this may, in turn, make them susceptible to impaired glucose metabolism[141,145,149].

The ability of the pancreatic β-cell to secret insulin is dependent on its structural and functional integrity along with the nutritional availability[150]. Consequently, protein deficiency in the maternal diet is a definite contributor to reduced insulin secretion and decreased β-cell proliferation in low protein programmed animals[151]. The reduced islets area and β cell number are mainly due to the downregulation of genes *FoxO1* and *Pdx1* genes, or altered expression of Reg1 pathway genes[151-155]. Epigenetic regulation of Hnf4a expression and expression of microRNAs such as miR-15b, miR-199a-3p, and miR 342, and signaling of mTOR in islets of the progeny also found to be associated with low protein-induced beta-cell dysfunction[62,63,156]. Further, a maternal low protein diet demonstrated greater β-cell apoptosis rates and deviates the equilibrium of islet's apoptosis and replication in the offspring[157-159]. The pancreatic islet cells of these offspring exhibited greater oxidative stress and mitochondrial dysfunction[72,160]. Consequently, lower β-cell reserve, β-cell dysfunction and impaired mitochondrial function in islets may drive towards T2D later in life[62,72,86,155]. With the multiple pathways controlling β-cell functions are modulated by maternal low protein, it is reasonable to hypothesize that the low protein exposure predisposed the offspring to lean T2D[127]. A list of key genes involved in low protein programming is compiled in Figure 1.

A balanced *in utero* nutrition is essential for the normal development of the reproductive system. Epidemiological studies in humans and experimental in studies animals show the low protein/unbalanced diet *in utero* severely impacts the development of reproductive organs, sexual maturation, and reproductive function in the offspring[161-164], resulting in decreased testis weight, reduced Sertoli cell numbers, and late-onset of spermatogenesis in males[164-166]. Moreover, the classical male fertility markers, sperm count, and serum testosterone were also diminished in the offspring of low protein exposed mothers[163,164,167]. Similarly, the low protein programmed female offspring were found to be with compromised follicle development and follicle health[168,169]. The numbers of primordial, primary, and secondary follicles were significantly reduced along with abnormal estrous cycle and redox homeostasis[170-172]. The thyroid hormone production and hypothalamic-pituitary-gonadal axis are also found to be affected by maternal low protein diet[173,174]. The impaired reproductive function of offspring may be due to the altered expression of genes associated with steroidogenesis, folliculogenesis, and steroid hormone receptors in gonads[175-178]. In addition, changes in the hypothalamic-pituitary-gonadal axis to low protein may have adverse effects on the normal development and function of gonads[168,179].

The hypothalamus of the brain plays a critical role in glucose homeostasis by controlling hepatic glucose production and peripheral glucose utilization. Therefore, functional, or structural alteration of hypothalamic neurons may often lead to the onset of T2D[180,181]. The low protein programmed progenies have exhibited structural and functional changes in the neuronal centers, hypothalamic nuclei which regulate metabolism and body weight[102,181]. In addition, maternal low protein can also differentially affect the hypothalamic-pituitary-gonadal axis depending on the timing of the impaired nutrition. Early gestational nutrition impairment has been shown to make the pituitary more sensitive to GnRH, resulting in reduced reproductive function[182]. Further, it also alters hypothalamic-pituitary-adrenal axis function by deregulating corticosterone-inducible enzymes and associated enzyme receptors[183]. Other reports also show that brain sparing may not be as effective during *in utero* low protein exposure leading to compromised brain development in the offspring along with long-lasting deterioration in cognitive and motor functions[184,185].

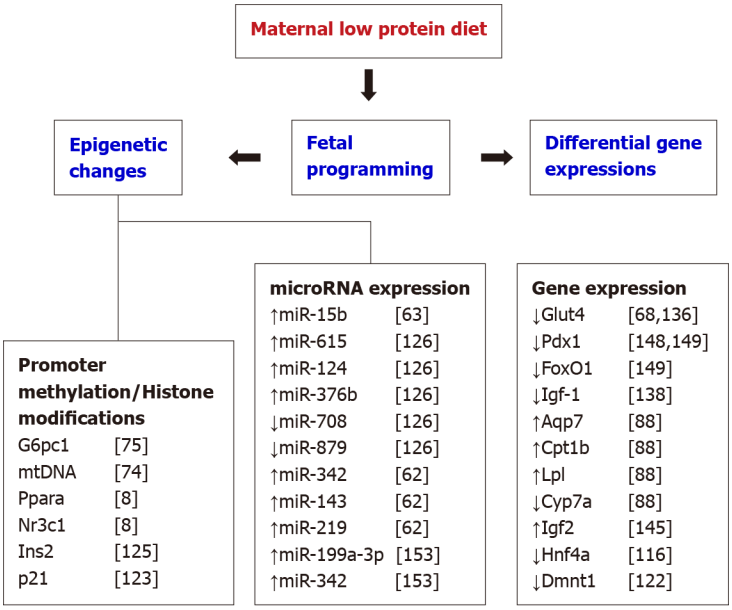

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Figure 1 Key gene expression and epigenetic changes observed in different maternal low protein studies.

PREVENTION AND TREATMENT

Insulin resistance and glucose intolerance are the cardinal signs of T2D, and if not prevented, they may lead to severe diabetes complications later in life. Hepatocytes, skeletal muscles, and adipocytes are the major insulin-dependent tissues that participate in the disposal of peripheral glucose. Thus, improving the muscle sensitivity towards insulin and enhancing hepatic glucose homeostasis, along with managing body weight are the central focus of T2D treatment strategies. Among the different drugs that have been prescribed for leanT2D management, metformin is a widely used drug for treating lean T2D along with nutritional and lifestyle modification[186]. Over the past two decades, various randomized control trials conducted in many ethnic groups showed unambiguously that the prevention is feasible by drugs or lifestyle modification[187-190].

Most of the research on treatment or prevention of T2D has been done with obese individuals or animal models, even though 10%-16% of all T2D people have normal BMI. In addition, majority of the studies on the molecular mechanisms of prevention and reversal of T2D were performed in Caucasians. Consequently, it is essential to include other ethnic groups such as Southeast Asian and Chinese populations, which are more prone to diabetes at lower average BMIs or lean T2D compared with white Europeans[191]. Regulating body weight is critical in the management of T2D associated with overweight or obese patients. However, in the case of leanT2D, it seems that leaner patients have severe beta-cell failure than normal-weight patients[4]. Presently, it is not clear that the achievement of lower body weight will help to prevent or reverse the special variants of T2D such as lean T2D[3].

The first trial associated with lifestyle modification and/drug therapy was started in China with a follow-up period of 23 years[192] and many other studies have followed since. Other studies include: the American diabetes prevention program outcome study[193]; the Finnish diabetes prevention study [194]; and the Indian short message service study[195] revealed the influence of lifestyle modification can persist long after the termination of the active phase of the trial. Although lifestyle modifications have been recognized to be very effective, safe, and ideal strategy for prevention, the effectiveness of relative risk reduction through these strategies exhibited some variations among different ethnic populations[192-196]. A study conducted among the impaired insulin tolerant lean Indian population found that lifestyle modification alone prevented the diabetes onset, regardless of relatively low BMI and highly insulin-resistant characteristics of the population[197].

Although the underlying pathophysiology of lean T2D is not completely understood, many studies using the maternal low protein model have shown potential prevention approaches[157]. The most promising approach among them is associated with one-carbon metabolism and the molecules involved in it. Some studies have reported the effectiveness of folic acid supplementation as a preventive treatment against the adverse effects of fetal programming[198-200]. Similarly, Burdge and team reported that the folic acid supplementation reversed the maternal low protein-induced phenotype epigenetically in the offspring when treated during the juvenile-pubertal period[201]. In contrast, our study reported a partial inhibition of gestational low protein-induced glucose intolerance only in female rats, when the maternal low protein diet was supplemented with folic acid from day 4 of the pregnancy until delivery[65]. Similar to our data, Lillycrop and colleagues also reported the inefficiency of folic

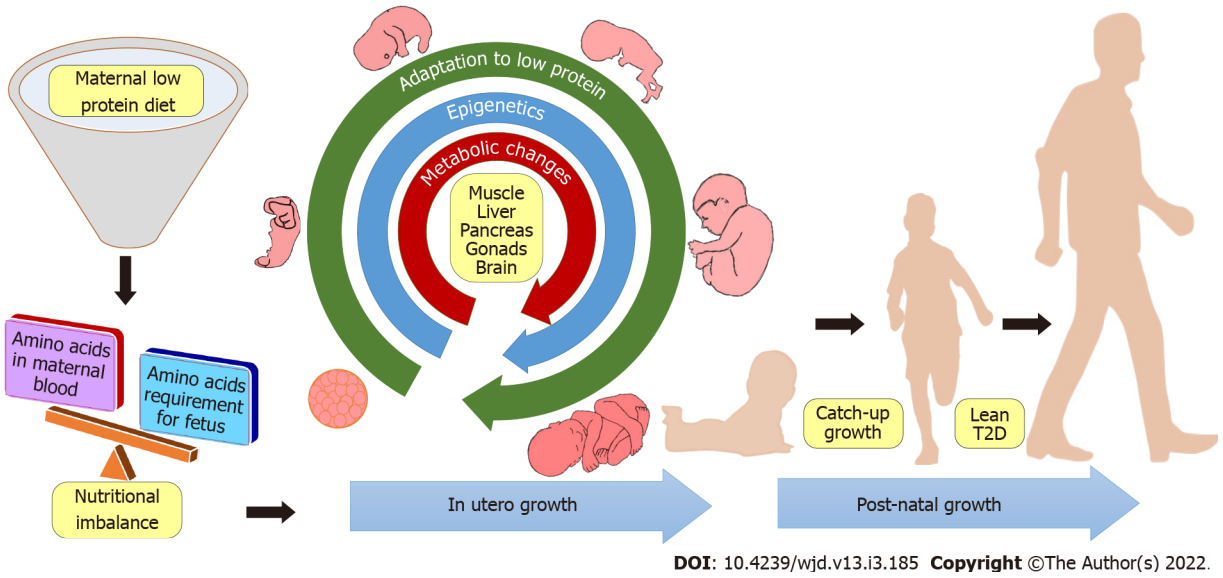

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Figure 2 Proposed mechanism of maternal low protein associated lean type 2 diabetes.

acid supplementation for the inhibition of gestational low protein-induced change in gene profile, although they found changes in the expression of genes associated with redox homeostasis[8]. An observational study from Pune, India (Pune Maternal Nutrition Study), noticed that when the mother was vitamin B12 deficient, high amount of folic acid intake was not enough to prevent the insulin resistance in the offspring[7]. However, the high protein to carbohydrate ratio in maternal diet was found to be effective in maintaining glucose homeostasis in the offspring[137]. Thus ensuring sufficient protein in the maternal diet is essential to prevent lean T2D.

CONCLUSION

In summary, lean T2D is a discrete subgroup of T2D with a set of specific clinical profiles. Atypical characteristics of leanness associated with insulin resistance needed to be dissected further for a better understanding of the etiology of the disease. As the progression of T2D may take many years in humans, the assessment and prevention studies with human subjects may also warrant many years. Therefore, the development of a well-defined animal model, which mirrors not only the pathophysiology of lean T2D but also the etiology of the disease, might be the most important step in this area of research. Nevertheless, there is a lack of a single animal model that can constitute all pathophysiological and etiological changes similar to humans. In addition, the severity of lean T2D is different between sexes, due to sex hormones and sex dependent expression of genes. Among different molecular mechanisms involved in the onset of lean T2D, the epigenetic underpinning of metabolism appears to be the most promising lead. Although the mechanism of developmental programming is currently not well characterized. With the current literature, it may be summarized that maternal low protein diet leads to diminished essential amino acids levels in the maternal circulation and consequently to the fetus. In such low protein environment, fetus is acclimatized and revises its growth and metabolic set points. This adaptation is thought to be due to the overall alteration of epigenetic and metabolic attributes of fetal energy homeostasis. Although these adaptations may be beneficial for the fetus, a nutritional mismatch with protein abundance in the adulthood often leads to metabolic derangements leading to diseases such as lean T2D. This concept is summarized in Figure 2. A better understanding of the molecular mechanisms of the disease may pave the way for more effective preventive and treatment strategies.

Obesity associated T2D is a serious public health problem throughout the developing and developed countries whereas nutritional deficiency especially protein deficiency is a major concern in under developed and developing countries. With studies showing a link between maternal protein consumption and T2D in offspring, it is essential to probe further and take action to avert a global crisis. Public health measures to alleviate poverty and access to nutritious and protein rich diet during pregnancy is essential to prevent lean T2D. Scientific understanding of the disease to prevent and treat T2D, along with effective health education and public policies can mitigate this growing global epidemic.

FOOTNOTES

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MINIREVIEWS

Can the management of depression in type 2 diabetes be democratized?

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Abstract

Both type 2 diabetes and depression are common and are projected to increase. There is increasing evidence for a bidirectional relationship between the two. Diabetes is a risk factor for depression; contrariwise, individuals with depression are at greater risk of developing diabetes. They are a burden for both the individual and the society. Co-existent depression worsens diabetic control because of obesity, insulin resistance and the adverse metabolic effects of anti-diabetes medicines. In addition, compliance to lifestyle measures required for diabetes is also compromised such as following a specific diet, taking proper medications on time, getting metabolic parameters assessed and maintaining a sleep cycle. Depression occurs in many grades; mild depression is more common in diabetes than frank or full-blown depression leading to suicide. Unfortunately, there are not enough trained and accessible mental health professionals such as psychologists or psychiatrists to deal with the increasing burden of depression in diabetes. Therefore, alternate models for management of mild to moderate depression are required. There is evidence that a team-approach by employing health care assistants can lower the risk of cardiac risk factors. INtegrating DEPrEssioN and Diabetes treatmENT study was carried out to determine whether the team-approach using non-health care professionals could be effective in managing mild to moderate depression and to study its effects on metabolic parameters among subjects with type 2 diabetes mellitus. The international study, carried out in four independent centers in India assessed the impact of a trained but not qualified non-psychiatrist in coordinating and forming a fulcrum between the patient, the family and the consultant endocrinologist/diabetologist. The interventions were fine-tuned to be culturally appropriate by qualitative interviews before they began. It was shown that the outcomes of both depression and diabetes could be improved by the employment of a clinical care coordinator. It is possible to scale up the studies to wider geographical areas and health-care organizations.

Key Words: Insulin resistance; Bidirectional; Patient health questionnaire-9; Care-coordinator; Antidepressants; Integrating DEPrEssioN and Diabetes treatmENT study; Non-professional

Core Tip: Type 2 diabetes and depression frequently co-exist. The presence of one worsens the outcome of the other. There are insufficient qualified professionals to treat depression. The INtegrating DEPrEssioN and Diabetes treatmENT study has shown that care-coordinators, who are trained but not professionals in mental health care can integrate and liaison among the patient, the family and specialists in treating mild to moderate depression associated with diabetes. Deployment of care-coordinators improved the outcome of depression and diabetes. This proof-of-concept study can be expanded and if found useful, help in democratizing the management of depression in diabetes.

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INTRODUCTION

The prevalence of type 2 diabetes is growing worldwide with depression rapidly following. Though it is possible that two common conditions can co-exist independent of one another, there is increasing evidence that diabetes and depression are related pathophysiologically, sharing a bi-directional relationship. When depression and diabetes coexist, the quality of life, compliance to treatment and outcomes are poor. Qualified specialists to manage diabetes and depression are in short supply. Therefore, innovative approaches are necessary to improve the outcomes of both. Based on published data between 1990 to 2016, it was estimated that among those aged 18-99 years, there were 451 million people with diabetes. By 2045, these were projected to increase to 693 million[1]. Of those with diagnosed diabetes, there is a greater prevalence in urban rather than rural (10.8% *vs* 7.2%) areas, and in high-income than in low-income countries (10.4% *vs* 4.0%)[2]. Mental disorders accounted for 13% of the global disease burden; major depression is projected to be the chief contributor to mental disorders by the year 2030[3]. Depression is commonly seen in other chronic illnesses also[4]. Multifactorial etiology of diabetes[5] and depression[6] requires multi-pronged management strategies.

COEXISTENCE OF DIABETES AND DEPRESSION

Diabetes mellitus is not a homogenous condition but results from a variety of pathogenic factors which are not always exclusive[7]. However, for clinical purposes, diabetes is classified into (1) Type 1 diabetes due to autoimmune destruction of the pancreatic β-cell leading to absolute insulin deficiency; (2) Type 2 diabetes mellitus having insulin resistance and a progressive loss of β-cell insulin secretion; (3) Gestational diabetes; and (4) Other specific causes leading to diabetes[8]. It is evident that psychological reactions differ in each of the different varieties of diabetes. In this presentation, management of depression is focused on type 2 diabetes, which is more common.

Twice as many people with diabetes are likely to have depression compared to the general population[9,10]. Hypertension, which is common in diabetes is associated with risk of depression and anxiety[11]. Resultantly the association of depression and diabetes has been the most commonly studied for the longest time[12]. A meta-analysis showed that compared to those with normal glucose tolerance, depression was more common in people diagnosed with diabetes; it was not high in those with pre-diabetes or those with normal glucose tolerance[13].

The number of prospective studies on the course of depression among people with diabetes is small; a meta-analysis of 11 follow up studies showed that type 2 diabetes subjects have a 24% increased risk of developing depression compared to controls[14]. Similarly, people with depression have a 32% increased risk for developing type 2 diabetes mellitus[15].

The grades of anxiety and depression associated with diabetes vary from subclinical depression to diabetes distress, which refers to emotional distress resulting from living with diabetes, a chronic non-remitting disease[16]. There are serious clinical implications when depression coexists with diabetes: The quality of life is impaired; the risk of morbidity and death is also increased. Operating factors include poor health care behavior which affects dietary habits, treatment, compliance to treatment,

motivation and productivity[16]. Long term diabetic complications are more common with comorbid depression[17]. Finally, the impact of combined diabetes and depression on quality of life is significant. Healthcare costs of managing type 2 diabetes associated with depression is higher than that of diabetes without depression[18]. Depression in type 2 diabetes can be treated[19], which improves the quality of life[17]. One must distinguish depression from diabetes distress. Diabetes distress is an emotional response to having diabetes, specifically the restricted lifestyle with having to follow self-management and the potential of complications in the long term[20]. Diabetes distress is associated with lessened self-care, and poorer emotional well-being, which, if left untreated may progress to severe depression[21]. Diabetes distress is far more common than clinical depression and is associated with poorer glycemic control[22]. The poor outcome is mediated in part by perceived control over diabetes such as one's innate ability to influence the course of diabetes[23].

Unlike the diagnosis of diabetes mellitus, depression is identified by clinical features such as episodes of lowered mood, reduced energy and decreased activity[8].

At the other end of the diabetes and depression spectrum is suicidality. Depression in persons with diabetes increases the risk of suicidality[24]. One must be aware of the risk factors for suicidal ideation and suicidal behavior, such as insulin administration, long duration of diabetes and poor glycemic control[25]. Identification and preventive measures are therefore essential in subjects with diabetes having depressive symptoms. Interventions must not only consider medical treatment, but also social factors associated with them[11], pointing to the need for integrated management processes.

COMMON PATHOGENESIS OF THE TWO CONDITIONS

Epidemiological studies have shown a bi-directional association between diabetes and depression[26]. Mendelian randomization studies have provided evidence that type 2 diabetes mellitus can cause depression: Single-nucleotide polymorphisms that predispose to diabetes predicted symptoms associated with depression[27]. Xuan *et al*[27] used 34 T2D risk genetic variants validated in East Asians as the instrumental variable (IV). An analysis using Mendelian randomization was carried out on 11506 participants from a prospective study. The diabetes genetic risk score (GRS) was built employing the 34 T2D common variants. The GRS was associated with depression even after adjusting for variables including age, sex, body mass index, current smoking and drinking, physical activity, education and marital status. A causal relationship was also found between genetically determined T2D and depression[27]. In addition, the stress associated with a new diagnosis of diabetes can precipitate depression[28].

The *common soil hypothesis* posits that factors common to both conditions could be the link for their association such as chronic inflammation, sedentary habits leading to obesity as well as vascular dysfunction[10]. Conceptually, the factors relating to both can be considered at two levels: *Behavioral* and *biological*[12]. Behavioral components include the burden of dealing with a chronic non-remitting disease and resultant poor lifestyle behavior. Sedentary lifestyle is a risk factor for depression[29], just as it is for obesity and diabetes mellitus. Biologically, hyperglycemia, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, chronic low grade inflammatory response and vascular dysfunction could all contribute. These are common to both diabetes and depression and may contribute to their co-existence[12]. There is evidence that behavioral and environmental factors are more responsible than genetic factors[12]. Vascular changes in small vessels supplying blood to the cerebral cortex are found in depression[30], although confirmatory studies are required. Brain-body dysfunction may contribute by impaired HPA regulation and by brain-gut microbiome axis[12]. Similarly, social stress can operate through epigenetic factors that activate the inflammatory response which is common to both diabetes and depression[15]. Use of some antidepressant drugs is also implicated in the risk of obesity, insulin resistance and diabetes mellitus[24,31].

Inflammatory changes, which occur in obesity, insulin resistance and diabetes mellitus occur in depression as *neuroinflammation*, involving activated microglia, astrocytes and oligodendroglia. These release mediators such as cytokines and chemokines, which when persistent, cause neurotoxicity[32]. Chronic inflammation in turn leads to insulin resistance and endothelial dysfunction, which has also been described in depression[32]. Hormonal components in women may contribute to gender differences in pathophysiological changes involving dysregulation of HPA and AN systems acting *via* immune and hemostatic pathways[33]. There is a flattening of the diurnal curve of the stress hormone cortisol which is associated with insulin resistance and could thereby play a role in the coexistence of diabetes and depression[34]. Along with abdominal obesity and insulin resistance, hypercortisolemia induces changes in glucocorticoid receptor-rich brain areas such as the hippocampus, amygdala and prefrontal cortex, where emotions and cognition are mediated[34].

Conceptually the relation between diabetes and depression can be considered in terms of (1) Psychological burden of a chronic disease such as diabetes predisposing the patients to depression and poor self-care behavior; (2) Diabetes and depression are coincidental, sharing common environmental and lifestyle factors; and (3) The cognitive behavioral construct attributes the burden due to diabetes leading to negative thoughts about diabetes in turn resulting in poor self-care behaviors[10]. Biological

underpinnings consist of one or a combination of (1) Activated immunity and inflammation mediated by cytokines; (2) Activation of HPA *via* stress; (3) Insulin resistance; (4) Disturbances of circadian rhythms; and (5) The contribution of antidepressant medications used in the treatment of depression[10] (Table 1).

Screening and diagnosis of depression

The diagnosis of diabetes, based on quantitative measurement of plasma glucose is far more refined than the diagnosis of depression; there are no biological markers for diagnosing depression. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) considers a major depressive episode as being present when at least five of nine symptoms suggestive of depression are present for 2 wk or longer; one of the nine must be a core symptom[12]. As a screening method for depression in diabetes, the Center for Epidemiologic Studies Depression Scale (CES-D) and Patient Health Questionnaire (PHQ)-9 were used most often in diabetes research[12]. Other screening tools for depression include Beck Depression Inventory, WHO well-being index and EDD[9].

Due to the non-specific nature of the symptoms and their overlap with uncontrolled hyperglycemia, the accuracy of screening tests varies between populations. Diabetes specific questionnaires are available to identify various psychological stresses[35]. There are some clinical pointers to distinguishing depression arising from diabetes and primary depression: The latter is suggested by mental disorders even before the diagnosis of diabetes, disproportionate symptoms compared to objective signs, a focus primarily on somatic symptoms and reassurance failing to relieve innocuous symptoms [36]. When these are inconclusive, screening for depression must be repeated after uncontrolled hyperglycemia is corrected[37]. However, caution must be exercised that affective symptoms such as pessimism or crying spells are not mistakenly attributed to poorly controlled diabetes[37].

For a rigorous diagnosis of depression, results of screening tests must be confirmed by a structured clinical interview such as SCID, Montgomery-Asberg Depression Rating Scale and the Composite International Diagnostic Interview[9]. These take time and require trained healthcare professionals, which limits the scope for practical application in routine clinical practice.

The reason for highlighting these aspects is to put in focus that the diagnosis of depression is subjective unlike the diagnosis of diabetes mellitus. Considering the subjective nature of diagnosing depression and the potential for false positive results, some national guidelines have not recommended population screening for depression[38]. A systematic review of screening tools for measuring depression in diabetes has shown that little data is available on their validity and reliability, with even lesser evidence for their being culturally appropriate[39]. In general, screening for major depressive disorders is based on screening instruments which do not generally consider the conceptual basis of emotional models, although efforts are being made to improve it[40]. Apart from the risk of false positive diagnosis of depression by assessing subjective methods, the outcomes of different methods of psychotherapy are not clear. The latter is being addressed by an ongoing trial: cRCT PSYCHOnline-THERAPY[41].

A Joint Position Statement of the American Diabetes Association, the American Association of Diabetes Educators, and the Academy of Nutrition and Dietetics, The American Diabetes Association recommends screening for depression in subjects with diabetes mellitus[42]. Others are advised an annual screen during major disease and life transitions.

Intervention strategies

In general, depression associated with diabetes can be managed by one or more of the following methods: Antidepressant drugs, psychological interventions such as cognitive-behavioral therapy, mindfulness-based cognitive therapy and stepped care[12].

Interestingly, many interventions that are useful in preventing and treating diabetes are also effective in depression. Physical exercise, including running helps in managing depression and other negative psychological conditions, although no quantitative measures are available to prescribe the quantum of exercise for its beneficial effects[43]. Insomnia, which often occurs with depression, is a well-known modifiable risk factor for the development of obesity and diabetes mellitus[44]. Cognitive behavior therapy for insomnia (CBT-I) is effective in improving insomnia associated with depression. CBT-I seeks to replace wrong beliefs of sleep, to help them prevent associating with stimulating activities, to limit time in bed for matching perceived sleep duration, sleep hygiene and relaxation techniques[45]. To ensure access to physical exercise and help in relaxation and ensuring adequate sleep, aspects of built environment must be considered[46].

One must recognize that guidelines for the management of depression are currently inadequately planned, reported and measured[47]. Therefore, shared decision making with the patient[48], using digital medical interview assistant systems at the primary care level could be employed to improve compliance and thereby management outcomes[49].

Although antidepressant medications are effective in the treatment of depression associated with diabetes, attention must be paid to their potential role in leading to obesity and insulin resistance[31]. Selective serotonin reuptake inhibitor agents (SRRI) are the drugs of choice, while considering the potential risk of hypoglycemia; should tricyclic antidepressants be required, one must carefully monitor glycemic control[50]. Along with antipsychotic medicines anti-depressants lead to weight gain which

Table 1 Links between type 2 diabetes and depression

Links	
Genetic	SNPs predisposing to diabetes predict symptoms associated with depression[27]
Common soil hypothesis[10]	Chronic inflammation
	Sedentary habits leading to obesity
	Activation of hypothalamic-pituitary-adrenal axis
	Disordered circadian rhythm
	Vascular dysfunction
Coincidental occurrence of both[10]	Sharing common environmental and lifestyle factors

ranges from 0.43 to 4.45 kg, with its attendant adverse metabolic effects through weight gain itself or its effects on the pancreatic beta cells. Dyslipidemia may result from the use of valproic acid derivatives, carbamazepine, mirtazapine. SSRIs can lead to dyslipidemia. Clozapine, olanzapine, valproic acid derivates and tricyclic antidepressants are known to induce insulin resistance and diabetes mellitus[51]. Newer agents such as bupropion and agomelatine, although promising, need more evidence for their therapeutic utility. Pharmacological agents used along with psychotherapy could prove to be more effective than either alone.

In a meta-analysis of 14 randomized clinical trials involving 1724 subjects, van der Feltz-Cornelis *et al* [52] concluded that treatment can improve clinical outcomes, although the combined effect of all interventions is moderate on the clinical impact[52]. When combined with diabetes self-management, psychotherapeutic interventions have a moderate clinical impact. Employing collaborative care *via* stepped care intervention is possible at the primary care level. Drug therapy and collaborative care successfully reduced depressive symptoms but did not have a significant effect on glycemic control[52].

Constraints of treating depression in diabetes

While the association between diabetes and depression, as well as the need for managing both together are recognized, implementation faces many barriers. As alluded to earlier, the diagnosis of depression is a work in progress; the burden of diabetes is so overwhelming that the identification of depression gets diluted due to lack of both time and knowledge[52]. Considering depression and diabetes are best treated together, effective management requires an embedded integrated approach rather than treating each independently[9]. It is imperative that new treatment paradigms must be identified, developed and applied to manage the twin problems of diabetes and depression, *i.e.* to democratize the treatment processes.

INTEGRATED CARE OF DIABETES AND DEPRESSION

Primarily, studies on interventions for depression showed that integrating mental health treatment to primary care settings is possible through collaborative care[53]. The key component of the collaborative care model is care managers, who are non-physicians, often nurses or social workers. Under the supervision of a physician and a psychiatrist, they identify depression by using screening tools and further provide problem-solving therapy[53]. Although encouraging in principle, a number of practical limitations remain for its wider applicability.

Compelling evidence is building up for efficacy of collaborative care in improving both glycemic control and outcomes of depression treatment[54]. Improvement of glycemia operates through better compliance to treatment[42]. It remains to be seen if the collaborative care model can be implemented at the primary care level without the need for significant additional resources. Larger studies involving cost-effective outcomes are required to determine the feasibility of such approaches[53]. Similar conclusions were drawn in a systematic review and meta-analysis on the effect of collaborative care in subjects with depression and diabetes mellitus[55,56]. From eight studies which included 2238 patients, collaborative care improved response to treatment of depression, remission of depression and better compliance to medications (anti-depressants and anti-diabetes drugs); however, there was no significant improvement of glycemic control as assessed by glycosylated hemoglobin[56]. Collaborative care involves coordination among physicians, nurses, other specialists and professionals providing management specific to the patient using evidence-based guidelines.

INtegrating DEPrEssioN and Diabetes treatmENT study

The INtegrating DEPrEssioN and Diabetes treatmENT (INDEPENDENT) Study was carried out[57] to assess whether it would be possible to bridge the gap between the high prevalence of depression in

diabetes and lack of qualified psychiatrists. It was a collaborative care model involving care coordinator, endocrinologist/diabetologist and psychiatrist in four centers in India. It assessed whether depression, identified by PHQ-9 can be managed by care coordinators, who are not professional psychiatrists, but were trained to identify and help solve issues of treatment compliance and coping with stresses. Coordination was carried out with the family and with the other members of the healthcare team of the primary physician, endocrinologists/diabetologists and psychiatrists. This follow up study was carried out in four sites in India: Madras Diabetes Research Foundation, Dr. Mohan's Diabetes Specialties Centre, Chennai, Department of Endocrinology, AIIMS, New Delhi, Endocrine and Diabetes Centre, Visakhapatnam, Diacon Hospital, Bangalore. The primary aim was to see whether there would be an improvement in depressive symptoms and metabolic parameters and whether they would be sustained for 12-mo after active intervention[58]. In the parallel, open-label, pragmatic randomized clinical trial (n:196 intervention group; n:208 controls), those who were in the intervention group were given 12 mo of support for self-management by nonphysician care-coordinators, decision support based on electronic medical records, under the periodic reviews by endocrinologists/diabetologists and psychiatrists. After a further 12-mo period of follow up without intervention, the outcomes were assessed. Control subjects received usual care for 24 mo[58]. Collaborative care intervention led to improvements in composite measure of depressive symptoms and indices of cardiometabolic health at the end of 24 mo [45].

Treatment aspects were obtained from published literature which were further adapted to local conditions by qualitative interviews involving patients with diabetes and their significant others[59]. To assess adaptations that were made to behavioral intervention made by care coordinators, and how patients responded to them, a purposive sample of patients (n:62) and care coordinators (n:3) were recruited from two clinics. Patients were interviewed about their experiences in the care model and care coordinators were interviewed about their experiences in implementation of interventions[46]. The adaptations sought and made were categorized by how they helped to improve implementation in the local context. They in turn served to help improve communication of health and to enhance engagement by the patients[59].

The use of care coordinators in managing depression among subjects with type 2 diabetes has shown promising results a year following active interventions. Further follow up and replication in other settings should be carried out to assess the generalizability of the findings from INDEPENDENT study. Recently, anxiety was shown to respond favorably to interventions in the INDEPENDENT study[60].

CRITICAL SUMMARY OF TYPE 2 DIABETES AND DEPRESSION

Judging from the number of publications, one could draw an erroneous opinion that the relationship between depression in type 2 diabetes is fully established, that effective treatment options are available and that the only constraint is to scale up intervention strategies to manage depression and type 2 diabetes. At the outset there is an asymmetry in the diagnoses of both conditions: Whereas diabetes is identified by objective criteria involving measurement of biomarkers, the diagnosis of depression is based on subjective criteria. The results from self-administered questionnaires and expert face to face interviews often diverge, as do different forms of questionnaires. The sensitivity and specificity of questionnaires need to be refined by including the cultural contexts of different populations. Therefore, there is a spectrum of conditions of what is referred to as depression associated with type 2 diabetes, from diabetes distress to subclinical depression, stretching to full blown depression. Interventions improve the outcomes of depression and of diabetes distress; however, treatment of depression improves depressive symptoms, without significant improvement of metabolic control. In contrast, treatment of diabetes distress results in improved glycemic control. Furthermore, the measures to manage them are varied and there are no accepted standard methods, rendering comparisons difficult. Therefore, despite epidemiological and mechanistic evidence for the co-existence of depression and type 2 diabetes mellitus, further refinements are necessary to define and measure the outcome of different treatment modalities of depression. However, most studies report improvement of depressive symptoms with interventions despite equivocal or no improvement of glycemic control. Therefore, it is worthwhile to identify depression in type 2 diabetes mellitus, and provide treatment by psychological and pharmacological measures. Although depression has been shown to respond to treatment, care must be taken in the choice of anti-depressant medications, some of which can worsen insulin sensitivity leading to adverse metabolic consequences. There is a lack of qualified mental care specialists to deal with the burgeoning burden of diabetes and depression. The employment of trained clinical care coordinators is a worthwhile attempt to improve access to subjects with type 2 diabetes having coexistent depressive symptoms. Preliminary results suggest the efficacy of such interventions. Further studies must be carried out to scale up across different cultural, ethnic and geographic populations.

CONCLUSION

It is established that diabetes and depression often coexist and must be managed together rather than individually. Interventions must be made across a spectrum to prevent, identify and manage depression when it occurs. Proof of principle studies have shown that they are feasible. It is necessary to scale-up such studies to assess their feasibility for wide-spread use in terms of applicability, efficacy and in terms of cost-benefit outcomes.

Non physician trained clinical coordinators can provide self-management education and support in terms of nutrition, lifestyle, compliance to medications, monitoring of metabolic parameters and dealing with psychosocial problems. These must necessarily be adapted to the age group, culture and language of the population by making appropriate cultural changes in education[16]. Depending on the availability and applicability, online interventions can be profitably made in terms of digital medical interview assistant systems[36]. With the widespread use of electronic medical records in diabetes care, a rule-based system can be incorporated so that standardized collection of data can be streamlined[61]. As the next logical step, the data can be analyzed and machine-learning methods can be devised to improve the communication, care and outcomes of diabetes and its associated morbidities including depression[62].

FOOTNOTES

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MINIREVIEWS

Humanin and diabetes mellitus: A review of *in vitro* and *in vivo* studies

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Abstract

Humanin (HN) is a 24-amino acid mitochondrial-derived polypeptide with cyto-protective and anti-apoptotic effects that regulates the mitochondrial functions under stress conditions. Accumulating evidence suggests the role of HN against age-related diseases, such as Alzheimer's disease. The decline in insulin action is a metabolic feature of aging and thus, type 2 diabetes mellitus is considered an age-related disease, as well. It has been suggested that HN increases insulin sensitivity, improves the survival of pancreatic beta cells, and delays the onset of diabetes, actions that could be deployed in the treatment of diabetes. The aim of this review is to present the *in vitro* and *in vivo* studies that examined the role of HN in insulin resistance and diabetes and to discuss its newly emerging role as a therapeutic option against those conditions.

Key Words: Diabetes mellitus; Insulin resistance; Humanin; Aging; Apoptosis; Oxidative stress

Core Tip: Humanin (HN) exerts cyto-protective and anti-apoptotic effects. Type 2 diabetes mellitus (T2DM) is considered an age-related disease. Beyond the role of HN against age-related diseases, it increases insulin sensitivity, improves the survival of pancreatic beta cells, and delays the onset of diabetes. Altered HN levels could serve as a potential biomarker in prediabetes and T2DM, since they seem to be an effect or a response to the increased reactive oxygen species production, oxidative stress, and reduced mitochondrial DNA copy number-A major and important question is whether HN could be used as a potential therapeutic option for diabetes.

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INTRODUCTION

Twenty years ago, three independent laboratories discovered humanin (HN) (MTRNR2), the first mitochondrial small open reading frame (sORF)-encoded microprotein found to have biological activity. The Hashimoto laboratory discovered HN while searching for survival factors in the unaffected brain section of an Alzheimer's patient[1]. The investigators revealed a cDNA fragment that mapped back to the mitochondrial 16S rRNA. This microprotein was named humanin because it displayed protection against Alzheimer's disease (AD)-related neurotoxicity, an action that the original authors though potentially could retrieve the "humanity" of patients suffering from dementia. Second, Ikonen *et al*[2] found that HN bound insulin like growth factor binding protein 3 (IGFBP3) using a yeast two-hybrid screening system and intensified the protective effects of IGFBP3 against amyloid-β (Aβ) toxicity. Also, Guo *et al*[3] showed that HN can bind and suppress the apoptotic protein BAX and, subsequently, alleviate cell apoptosis.

Physiologically, HN is produced by tissues in several organs, including kidney, skeletal muscles, brain, heart, and liver[4-6]. Subsequently, it is secreted into the blood circulation and transported to various target cells, protecting in parallel cells against several diseases strongly associated with oxidative stress, mitochondrial dysfunction, and cytotoxicity[7]. Beyond the cytoprotection HN possesses a key role in cell metabolism and mediates the production and secretion of endocrine/paracrine/autocrine protective stress response factors[8]. Additionally, it plays a role in age-related diseases and several metabolic disorders (*e.g.*, cardiovascular diseases [CVD], memory loss, stroke, diabetes type 2 [T2DM]).

Diabetes is a chronic disease that occurs either due to autoimmune destruction of the pancreatic beta cells, leading to absolute insulin deficiency (T1DM) or due to progressive attenuation of insulin secretion on a background of insulin resistance resulting in relative insulin deficiency (T2DM). The number of people with diabetes rose from 108 million in 1980 to 422 million in 2014. Prevalence has been increasing faster in low- and middle-income countries than in high-income countries. The rising burden of T2DM is a major concern in health care worldwide. In 2017 6.28% of the worldwide population was affected by T2DM. It is disconcerting that the burden of the disease is rising globally, and at a more rapid rate in developed regions such as western Europe[9]. As for the T1DM, its incidence is estimated 15 per 100000 people and the global prevalence 9.5%[10]. Since diabetes and its complications affect individuals' functional capacities and quality of life leading to significant morbidity and premature mortality, effective agents are required for its treatment.

STRUCTURE OF HUMANIN PEPTIDE

HN is encoded by a sORF within the gene for the 16S ribosomal subunit in the mitochondrial genome [11]. HN has a positively charged N-terminal (Met-Ala-Pro-Arg), central hydrophobic region (Gly-Phe-Ser-Cys-Leu-Leu-Leu-Leu-Thr-Ser-Glu-Ile-Asp-Leu), and negatively charged C-terminal (Pro-Val-Lys-Arg-Arg-Ala)[1]. Last three amino acid residues in the C-terminal are considered as dispensable because both 21 and 24-amino acid long peptides have indistinguishable intracellular and extracellular effects [12]. Thirteen nuclear-encoded HN isoforms have been identified. HN-like ORF has been named MTRNR2L1 to MTRNR2L13 after the original humanin MTRNR2 gene in the mitochondrial genome. MTRNR2L1—MTRNR2L10 are expressed in most human tissues, with MTRNR2 being expressed in higher proportion in comparison to the other isoforms. Molecular manipulations of HN at key amino acids lead to changes in chemical characteristics. Additionally, single amino acid substitutions can lead to significant modifications to its biological functions and potency[13].

MECHANISMS OF ACTION

HN exerts its functions after connecting to either intracellular molecules or cell membrane receptors (Figure 1). Immediately after HN's receptor binding, extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation increases[14]. Once ERK1/2 is phosphorylated, it separates from its anchoring proteins, and transfers to other subcellular compartments. ERK1/2, a member of the mitogen-activated protein kinase pathway, participates in several essential cellular processes such as cell proliferation, survival, differentiation, mobility, and apoptosis[15,16]. HN behaves as a link to two different types of receptors: the seven-transmembrane G protein-coupled receptor formyl peptide receptor-like 1 (FPRL1) which plays a role in the cytoprotective properties of HN and a trimeric receptor, consisting of ciliary neurotrophic factor receptor (CNTFR), the cytokine receptor WSX-1 and the transmembrane glycoprotein 130 (GP130) (CNTFR/WSX-1/GP130) which is essential for HN activity and its neuroprotective effects[17]. As regards GP130, it is a transmembrane protein that acts as the signal transduction unit of the IL-6 receptor family[18]. Dimerization of GP130 receptors provokes the stimulation of janus kinases (JAK1 and JAK2), then subsequently provokes signal transducer and activator of transcription 3 (STAT3) and STAT1[19]. The dimerized STATs move to the nucleus and control transcription. The second signaling pathway directed by GP130 recruits SHP-2. SHP-2 is phosphorylated by JAK and interacts with growth-factor receptor bound protein 2 (Grb2), which allows the activation of mitogen-activated protein kinase (MAPK)[19].

HN is regulated by insulin-growth factor 1 (IGF-1) and growth hormone (GH). HN and IGF-1 Levels decrease with age[20]. It has also been suggested that GH inhibits HN levels *via* IGF-1. Treatment with GH or IGF-1 reduces circulating HN levels in both mice and human subjects[21]. To date, HN has been suggested to play a role in various diseases like T2DM[22,23], CVD[4,5], memory loss[24], amyotrophic lateral sclerosis (ALS)[25], stroke[26], and inflammation[12,27]. The main mechanisms that dominate and link these age-related diseases are oxidative stress[28] and mitochondrial dysfunction[29]. Mitochondria are principal sources of reactive oxygen species (ROS) which can cause oxidative stress and injure of the lipids, proteins, and DNA of the cells. This can afflict mitochondrial function, and, subsequently, enhanced ROS production occurs[29]. These circumstances contribute to cellular damage, apoptosis, and cellular ageing, causing ageing and age-related diseases such as Parkinson's disease[30], Alzheimer's disease[31], atherosclerosis[32], heart failure[33], myocardial infarction[34], chronic inflammation[35], kidney disease[36], stroke[37], cancers[38], and many kinds of metabolic disorders[39,40].

Especially concerning diabetes, HN provides protection against apoptosis by binding pro-apoptotic Bax, inhibiting its mitochondrial localization, and lessening Bax-mediated apoptosis activation[3], acting either directly on Bax or through the FPRL-1 receptor[17]. As for its neuroprotective action, which has also a place in the neuroendocrine beta cells protection, it involves HN binding to a complex involving CNTFR/WSX-1/GP130[17] and activation of tyrosine kinases and STAT-3 phosphorylation[41]. Moreover, an important mechanism of cell protection may be *via* interfering with Jun N-terminal kinase (JNK) activity[42]. Important is also the interaction between HN and insulin-like growth factor binding protein-3 (IGFBP-3) which prevents the activation of caspases[2]. Furthermore, an alteration at position [Gly14]-HN (S14G, HNG) seems to induce neurosurvival activity and a substitution of phenylalanine in the 6th position with alanine (F6A, F6AHN) changes the binding of HN to IGFBP-3 and enhances its main effect on glucose metabolism and insulin sensitivity[5].

ROLE OF HUMANIN IN THE PATHOGENESIS OF TYPE 1 DIABETES

The role of HN in T1DM has been scarcely investigated. T1DM is characterized by the loss of pancreatic beta cells which results in insulin deficiency. The beta cells destruction, the dominant event in the pathogenesis of T1DM, occurs as a result of the IL-1, TNF-a, and IFN- γ actions which are originated from T cells and macrophages. Since HN is identified as a survival factor[43], it seems to serve also as a survival factor for neuroendocrine beta cells by decreasing cytokine-induced apoptosis and subsequently, improves glucose tolerance and onset of diabetes as it has been demonstrated in NOD mice *in vivo*[44]. Yet, no studies juxtaposing the HN levels in T1DM and T2DM have been published thus far.

ROLE OF HUMANIN IN THE PATHOGENESIS OF TYPE 2 DIABETES

T2DM is one of the most common metabolic diseases. This metabolic disorder and its comorbidities and complications, such as CVD, stroke, chronic kidney disease (CKD), and cancer, are global health problems which, noticeably, diminish quality of life and life expectancy[45-48].

Mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of diabetes. Mitochondria are principal elements for the maintenance of metabolic health and cellular energy homeostasis. Mitochondrial dysfunction causes glycaemic dysregulation and metabolic derangement [49]. It causes inefficiency in the electron transport chain and beta-oxidation, thus trigging insulin

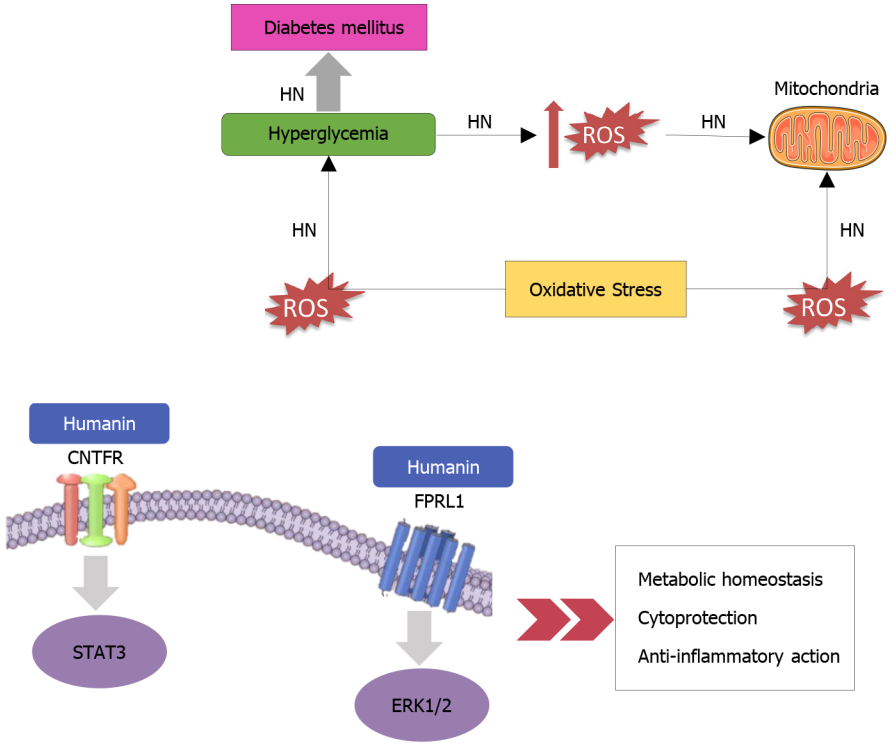

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Figure 1 Mechanisms of action of Humanin in diabetes mellitus. CNTFR: Ciliary Neurotrophic Factor Receptor; ERK1/2: Extracellular signal-regulated protein kinases 1 and 2; FPRL1: Formyl peptide receptor-like 1; HN: Humanin; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of transcription 3.

resistance[50]. Furthermore, hyperglycemia provokes ROS generation which, in turn, causes oxidative stress in several tissues, cellular lipids, proteins, and DNA, and subsequently, provokes chronic inflammation[51]. The accumulation of oxidative damage leads to a decrement of mitochondrial function which can result in increased ROS production[29]. It has been suggested that mitochondrial dysfunction is implicated in diabetes-related complications impairing the kidneys, nervous system, heart and retina, and that mitochondrial dysfunction-related oxidative stress contributes to these complications[52]. Subsequently, an increase in ROS concentrations may provoke HN mobilization from various tissues to the impaired areas, where HN acts against oxidative stress, decreases ROS production, and promotes cell survival[51]. Mitochondrial derived peptides (MDPs), such as HN, have been suggested to play a critical role in reducing oxidative stress[53-55] and improving T2DM[56]. It has also been demonstrated that HN promotes mitochondrial biogenesis in pancreatic β-cells[57].

IN VITRO AND ANIMAL STUDIES

In vitro and animal studies

Considering that diseases related with ageing, named T2DM and neurodegeneration, have been suggested to be associated with mitochondrial dysfunction[58,59], it follows that the mitochondrial-derived peptide HN regulates them (Table 1). Based upon the molecular interaction between HN and IGFBP-3, that prevents the activation of caspases, and since IGFBP-3, independent of IGF-1, provokes IR both at the liver and periphery[60,61], Muzumdar *et al*[23] hypothesized that HN, besides its neuroprotective action, may regulate glucose homeostasis. Utilizing state of the art clamp technology, they investigated the role and the mechanism of action of central and peripheral HN in glucose metabolism. They finally demonstrated that infusion of HN improves both hepatic and peripheral insulin sensitivity and that hypothalamic STAT-3 activation is essential for the insulin-sensitizing action of HN. Moreover, treatment with a highly potent HN analog significantly lowered blood glucose in Zucker diabetic fatty rats. As for the levels of HN in tissues like hypothalamus, skeletal muscle, and cortex, they reduced with age in rodents, and its' circulating levels were also diminished with age in humans and mice.

A year later, a group from California[44] investigated whether HN could improve the survival of beta cells and delay or even treat diabetes in NOD mice. HN prevented apoptosis induced by serum starvation in NIT-1 cells and decreased cytokine exposure-related apoptosis (caused by interleukin [IL]-1β, tumor necrosis factor [TNF]α, and interferon[IFN]γ). STAT3 is considered as a principal survival

Table 1 *In vivo* and *in vitro* studies on humanin and diabetes mellitus

Ref.	Study model	HN dose	Treatment duration	Results
In vitro studies				
Rochette *et al*[51], 2014 (HN)	NIT-1 cells	1-10000 nmol/L	24 h	Reduced apoptosis caused by serum starvation in NIT-1 cells and decreased cytokine-induced apoptosis
Hunter and Jones [19], 2015 (HNGF6A)	Isolated islets and cultured murine β cell line	50 ng/ml	15-120 min	Enhanced glucose-stimulated insulin secretion
Qin *et al*[57], 2018 (HNG)	HUVECs	1 μM	3 h	Inhibited cell death, nucleus pyknosis and deformation. Diminished the expression of cleaved PARP (which reflects the level of apoptosis as well as ROS) Decreased the level of bax (a pro-apoptotic protein). Increased bcl-2 (an anti-apoptotic agent)
Miller *et al*[11], 2020 (HNG)	HEK293 and SH-SY5Y cells	100 μM	30 min	Is a major GP130 agonist which acts through the GP130/IL6ST receptor complex and activates AKT, ERK1/2, and STAT3
Wang *et al*[50], 2010 (HN)	Pancreatic MIN6 β-cells	25, 50 and 100 μM	24h or48 h	Increased the expression of PGC-1α.Promoted mitochondrial biogenesis. Caused the phosphorylation of AMPK, improved mitochondrial respiration and stimulated ATP generation
Kim *et al*[60], 2007 (HN)	HUVECs	200 μM	24 h	Promoted the expression of KLF2.Reduced the expression of VCAM-1 and E-selectin; Impeded the secretion of TNF-α and IL-1β
In vivo studies				
Animals				
Hunter and Jones [19], 2015 (HNGF6A)	Sprague−Dawley rat	0.07 mg/kg/h	2-30 min	Improved insulin sensitivity and help in decreasing blood glucose level
Gong *et al*[20], 2014 (HN)	Sprague−Dawley rat	0.375 mg/kg/h	360 min	Decreased blood glucose in Sprague−Dawley rats by STAT-3 phosphorylation
Gong *et al*[20], 2014 (HNGF6A)	Zucker diabetic fatty rat	0.05 mg/kg/h	90-240 min	Decreased blood glucose in Zucker diabetic fatty rats
Rochette *et al*[51], 2014 (HN)	NOD mice	0.7 mg/kg/day	6 wk20 wk	Decreased lymphocyte infiltration in mice pancreata; Delayed or prevented the onset of diabetes in NOD mice (when the treatment was extended up to 20 wk)
Miller *et al*[11], 2020 (HNG)	Male C57BL/6 mice	5 mg/kg/day	2 wk	Old mice, but not young mice, showed an increase in phosphorylation in AKT and ERK1/2 in the hippocampus
Humans				
Muzumdar *et al* [61], 2006	Participants attending a diabetes complications screening clinic	-	-	A significant decrease in HN was observed in the IFG group compared to control
Ha[71], 2006	Uncomplicated T1DM patients	-	-	Plasma HN levels were significantly higher in T1D men by comparison with the healthy control men
do Nascimento *et al* [72], 2013	Pregnant women with and without GDM	-	-	Serum HN levels were significantly lower in women with GDM compared to controls
Hashimoto *et al*[42], 2003	Normal, prediabetes and diabetes subjects	-	-	Serum HN concentrations are lower in T2DM and correlate with HbA1c

HUVECs: Human umbilical vein endothelial cells; PARP: Poly ADP-ribose polymerase; ROS: Reactive oxygen species; ERK1/2: Extracellular signal-regulated kinase 1/2; STAT3: Signal transducer and activator of transcription 3; PGC-1α: PPAR-γ coactivator-1α; KLF2: Krüppel-like factor 2; VCAM-1: Vascular cell adhesion molecule 1; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IFG: Impaired fasting glucose; T1DM: Type 1 diabetes mellitus; GDM: Gestational diabetes mellitus.

signaling protein in beta cells, regulating the pro-survival effects of various growth factors and cytokines. HN activated STAT3 and ERK over a 24-hour time course. Interestingly, HN improved glucose tolerance in NOD mice and after 6 wk of treatment decreased lymphocyte infiltration was observed in their pancreata. When the treatment was extended up to 20 wk the investigators noted that HN delayed or prevented the onset of diabetes in NOD mice.

A few years later, the group we mentioned first[23] hypothesized that HNGF6A, a potent non-IGFBP-3 binding HN analog, may affect acutely and independently insulin secretion, since insulin concen-

trations were not reduced along with hypoglycemia caused by HNGF6A in Sprague Dawley rats[22]. Sprague Dawley rats that received HNGF6A presented higher insulin levels during hyperglycemic clamps compared to controls. Similarly, *in vitro*, HNGF6A enhanced glucose-stimulated insulin secretion in isolated islets and cultured murine β cell line. This effect was dose dependent, combined with ATP production in the β cell, related to the KATP-channel-independent augmentation phase of insulin release[62], and associated with amplified glucose metabolism. These potent effects on insulin secretion in combination with the effects on insulin action suggested a role of HN in the treatment of T2DM.

The protective effects of [Gly14]-Humanin (HNG) against high glucose-induced apoptosis were investigated in human umbilical vein endothelial cells (HUVECs). Pretreatment of HUVECs with HNG inhibited cell death, nucleus pyknosis and deformation[63]. Also, HNG diminished the expression of cleaved poly ADP-ribose polymerase (PARP) which reflects the level of apoptosis as well as reactive oxygen species (ROS). Regarding the level of bax, which is a pro-apoptotic protein, it decreased after pretreatment with HNG, while bcl-2, which exerts anti-apoptotic effects, it increased.

Another group identified a different sORF within the mitochondrial 12S rRNA encoding a 16-amino-acid peptide named MOTS-c (mitochondrial open reading frame of the 12S rRNA type-c) which also regulates insulin sensitivity and metabolic homeostasis[56]. Particularly, MOTS-c treatment in mice protected against age-dependent and high-fat-diet-induced insulin resistance and diet-induced obesity as well. Finally, they suggested that MDPs, like MOTS-c and HN, with such systemic effects may be useful in ameliorating the abnormal metabolism associated with aging in humans and regulating biological processes like weight and metabolic homeostasis.

Kim and his colleagues from California tried to elucidate the signaling pathways underlying HN'S cytoprotective roles *in vitro* and *in vivo*[14]. Utilizing multiple models, they showed that HN is a major GP130 agonist which acts through the GP130/IL6ST receptor complex and activates AKT, ERK1/2, and STAT3. PI3K, MEK, and JAK were suggested to be involved in the activation of those three signaling pathways, respectively.

Concerning the effects of HN on mitochondrial biogenesis in pancreatic β-cells, HN treatment in MIN6 β-cells increased the expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α)[57] which promotes mitochondrial biogenesis by activating the expression of nuclear respiratory factor 1 (NRF-1) and mtDNA transcription factor A (TFAM)[64]. Also, HN treatment promoted mitochondrial biogenesis by increasing mitochondrial mass, elevating mitochondrial DNA (mtDNA)/nDNA ratio (reduced mtDNA copy number plays a key role in insulin resistance[65]), and increasing cytochrome B expression. Finally, HN treatment resulted in the phosphorylation of AMPK, which was involved in the induction of PGC-1α, NRF-1, and TFAM and improved mitochondrial respiration and stimulated ATP generation leading to a possible functional gain of the mitochondria.

In HUVECs also, HN displayed protective action against high-glucose-induced endothelial dysfunction and macrovascular complications[66]. HN treatment promoted the expression of Krüppel-like factor 2 (KLF2), a principal transcriptional regulator of endothelial function, by activating ERK5. In addition, HN significantly reduced the expression of vascular cell adhesion molecule 1 (VCAM-1) and E-selectin, which regulate the adhesion of circulating leukocytes to the endothelium, a principal procedure in the initiation of atherosclerosis. Furthermore, HN impeded the secretion of pro-inflammatory cytokines, such as TNF-α and IL-1β.

HUMAN SUBJECTS RESEARCH AND CLINICAL TRIALS

Human subjects research and clinical trials

The first attempt to measure HN levels in a clinical population with impaired fasting glucose (IFG) was made in participants attending a diabetes complications screening clinic (DiabHealth)[67,68]. Previous clinical studies reported noticeably increased HN levels in patients with mitochondrial encephalomyopathy, lactic acidosis, and stroke−like episodes (MELAS) and chronic progressive external ophthalmoplegia (CPEO), which are associated with excess oxidative stress[69,70]. However, a significant reduction (P = 0.0001) in HN was reported in the IFG group (n = 23; 204.84 ± 92.87 pg mL^{-1}) compared to control (n = 58; 124.3 ± 83.91 pg mL^{-1}) in accord with an adaptive cellular response by HN to a slight raise in fasting blood glucose level (BGL). As we described above, HN protects neuroendocrine β-cells [44] and increases glucose tolerance and insulin sensitivity[20,44]. Moreover, it is considered to interact with hydrogen peroxide and α-actinin-4 which rise during oxidative stress and IFG[71-73] and binds extracellularly with the CNTFR/WSX−1/GP130 receptor[69,74,75]. Interestingly, mild to moderate levels of ROS result in positive adaptive mechanisms of the mitochondria[76]. All these mechanisms, which benefit cell function and survival, lead to a reduction in HN levels, indicating a protective role of HN. However, with disease progression to T2DM and further oxidative stress, mitochondria may upregulate HN levels as observed in studies of Alzheimer's disease and in those of MELAS and CPEO.

These conditions are related to extensive oxidative stress which is also a key feature of DM. Particularly, hyperglycemia causes extended free radical activity and mitochondrial dysfunction which induce oxidative stress and release more ROS[76]. The advanced diseases MELAS and CPEO are

associated with increased plasma HN levels. HN has a protective role and is upregulated with disease progression. On the contrary, the minor elevations of blood glucose levels are combined with a decrease in HN concentrations which supports the protective role of HN when levels are expected to decrease as a result of stimulation of oxidative stress-associated agents that are inhibited by HN. However, with disease progression to T2DM and further oxidative stress, mitochondria increase HN levels, as reported in MELAS and CPEO.

A few years earlier, another group from Toronto suggested that plasma HN levels were significantly higher in T1D men by comparison with the healthy control men ($P < 0.0001$)[77].

At the end of 2018 Ma *et al*[78] evaluated HN concentrations in pregnant women with and without gestational diabetes mellitus (GDM) aiming to define the role of HN in the development of GDM. 157 women were enrolled in the study. Serum HN levels were significantly lower in women with GDM compared to controls. Like Lee *et al*[21], who found that HN was regulated by IGF-1 in mice and humans, they suggested that the IGF axis influenced the HN levels and affected its normal function in GDM. By performing logistic regression analysis, they also showed that low HN levels were the independent risk factor of GDM and, therefore, might be a predictor for the GDM diagnosis. Additionally, HN levels were significantly negatively correlated with the body weight, body mass index (BMI) and homeostatic model assessment for insulin resistance (HOMA-IR).

The most recent study which attempted to evaluate MDP levels in normal, prediabetes and diabetes subjects enrolled 225 participants[49]. The investigators found that serum HN concentrations are lower in T2DM ($P < 0.0001$) and correlate with HbA1c. Interestingly, HN levels decreased by 62% in the prediabetes group, 66% in diabetes subjects with good control and 77% in uncontrolled diabetes patients compared to participants without diabetes. Also, this study confirmed that there are no significant differences in HN levels between healthy men and women and the levels of HN were not affected by the different anti-diabetic treatment (insulin, metformin, other hypoglycemic regimens) or the duration of therapy. Furthermore, since HN was associated with adiponectin, which has been suggested to be reduced in prediabetes and T2DM[79], it can be concluded that mitochondrial dysfunction contributes to glycemic dysregulation and metabolic effects in T2DM. Adiponectin levels were positively correlated with HN. Adiponectin concentrations decrease in pre-diabetes and DM[79]. It has also been demonstrated that adiponectin knockout mice have reduced mitochondrial content combined with insulin resistance[80]. In addition adiponectin may impair mitochondrial biogenesis[81]. Therefore, the affected mitochondrial function may arise from the low adiponectin levels.

As for the changes in HN levels with ageing, Voigt *et al*[67] showed that HN decreased with age among individuals attending a diabetes complications screening clinic suggesting a protective function of HN and this observation was consistent with a previous study among human and mice[23]. On the contrary, circulating levels of HN increase in age-associated diseases such as T2DM. With disease progression and additional oxidative stress, mitochondria may increase HN levels.

Besides the initial and principal lifestyle interventions for glycemic control in DM, currently, we have various oral and injectable pharmacologic agents at our disposal including metformin, thiazolidinediones, sulfonylureas, glucagon-like peptide 1 (GLP-1) receptor agonists, dipeptyl-peptidase 4 (DPP-4) inhibitors, sodium-glucose co-transporter 2 (SGLT-2) inhibitors, and insulin[82]. These medicines can be administered in various dosages and in many combinations in each patient diagnosed with DM. However, there is still room for additional new factors that could efficiently contribute to the management of the disease. Given HN's protective properties, it may represent a novel treatment option to decrease the cellular damage caused by diabetes. Altered HN levels in diabetes could serve as a potential biomarker. Nevertheless, no clinical trials investigating the effects of HN or its analogues (e.g. HNGF6a) administration have thus far been published, albeit it would be an innovative and promising breakthrough in diabetes prevention and treatment.

CONCLUSION

In summary, HN shows cytoprotective effects in many biological processes, including oxidative stress and apoptosis. Altered HN levels could serve as a potential biomarker in prediabetes and T2DM, since they seem to be an effect or a response to the increased ROS production, oxidative stress, and reduced mtDNA copy number which all contribute to IR[83]. However, further study is needed to define the role of age and other modifiable confounding factors, like fitness level, adiposity, other metabolic comorbidities, such as CVD, stroke, inflammation. Undoubtedly, the major and important question is whether HN could be used as a potential therapeutic option for diabetes, that could even replace the current diabetes mellitus treatment strategies soon. Towards this direction, further studies are needed to identify the contribution of HN in the metabolic dysregulation of T2DM.

FOOTNOTES

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ORIGINAL ARTICLE

Case Control Study

Functional annotation and enrichment analysis of differentially expressed serum proteins in patients with type 2 diabetes after dapagliflozin

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Abstract

BACKGROUND

Only 50% of patients with type 2 diabetes mellitus (T2DM) can control their blood glucose levels. Dapagliflozin is a selective inhibitor of sodium-glucose co-transporter 2 (SGLT-2) that improves the insulin sensitivity of the liver and peripheral tissues. Many studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection. Nevertheless, the mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differentially expressed proteins in the serum of T2DM patients have not been intensively explored so far.

AIM

To identify differentially expressed proteins associated with dapagliflozin treatment in patients with T2DM.

METHODS

Twenty T2DM patients [hemoglobin A1c (HbA1c) 7.0%-10.0%] were enrolled at The Affiliated Hospital of Inner Mongolia Medical University between January 1, 2017 and December 1, 2018. They received dapagliflozin (10 mg/d) for 3 mo, and the HbA1c < 7.0% target was achieved. The changes in clinical indexes were compared before and after treatments. Label-free quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. The identified differentially expressed proteins were analyzed using various bioinformatics tools.

RESULTS

Dapagliflozin significantly improved the clinical manifestation of the patients. There were 18 downregulated proteins and one upregulated protein in the serum samples of patients after dapagliflozin administration. Bioinformatics analyses, including subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations, were used to profile the biological characteristics of the 19 differentially expressed proteins. Based on the literature and function enrichment analysis, two downregulated proteins, myeloperoxidase (MPO) and alpha II B integrin (ITGA2B), and one upregulated protein, podocalyxin (PCX), were selected for enzyme linked immunosorbent assay validation. These validated differentially expressed proteins had multiple correlations with clinical indexes, including HbAc1 and fasting C-peptide.

CONCLUSION

Dapagliflozin has hypoglycemic effects and regulates the serum expressions of MPO, ITGA2B, and PCX, possibly contributing to the effects of dapagliflozin on oxidative stress, insulin resistance, and lipid metabolism.

Key Words: Type 2 diabetes mellitus; Dapagliflozin; Non-standard quantitative proteomics; Myeloperoxidase; Alpha II B integrin; Podocalyxin

Core Tip: This study aimed to identify differentially expressed proteins associated with dapagliflozin treatment in patients with type 2 diabetes mellitus. Changes in blood indexes were examined in 20 patients treated with dapagliflozin for 3 mo. Quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. Dapagliflozin has hypoglycemic effects and regulates the serum expressions of myeloperoxidase, alpha II B integrin, and podocalyxin, possibly contributing to the effects of dapagliflozin on oxidative stress, insulin resistance, and lipid metabolism.

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INTRODUCTION

With the aging global population and the increase in the prevalence of obesity, it is expected that type 2 diabetes mellitus (T2DM) will affect more than 381.8 million people worldwide in 2035[1]. In the United States alone, T2DM is projected to affect nearly one in three people by 2050[2]. T2DM manifests through the development of fasting and postprandial hyperglycemia, which is the primary contributor to numerous life-threatening complications and co-morbidities[3,4]. These alarming projections suggest an urgent need for the development and implementation of novel preventative and treatment strategies to fight the rise in T2DM prevalence worldwide[4]. Unfortunately, despite the best care, only 50% of patients with T2DM can control their blood glucose levels[5-7].

In recent years, the participation of the kidney in glucose metabolism and homeostasis attracted much attention, and this participation has begun to be explored in clinical studies[8]. The mechanisms mainly include the renal tubular reabsorption of glucose, largely dependent on the expression of sodium-glucose co-transporter 2 (SGLT-2) localized at the proximal small tubules S1 and S2[9]. Dapagliflozin is a selective inhibitor of SGLT-2, reducing the reabsorption of SGLT-2 receptor glucose in renal tubular epithelial cells and allowing excess glucose to be excreted in the urine[10]. Thus, the insulin sensitivity of the liver and peripheral tissues can be improved, and the hepatic glucose output can return to the normal range[10,11]. Furthermore, many investigators proposed that SGLT-2 inhibitors have renal and cardiovascular protective roles in addition to their glucose-lowering effects[12-14]. Thereby, dapagliflozin is recommended for T2DM patients[15].

Several studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection[13-15]. Powell *et al*[16] suggested that SGLT2 inhibitor alone could reduce hemoglobin A1c (HbA1c) by 0.37%-1.16%. Several randomized, double-blind, controlled trials have confirmed that dapagliflozin can significantly reduce HbA1c (by up to 1.16%) and blood glucose and that the efficacy of dapagliflozin (10 mg) in reducing HbA1c is

comparable to that of metformin sustained-release tablets (2000 mg)[17,18]. Ji *et al*[19] proposed that SGLT2 inhibitors can reduce blood glucose and hyperglycemic toxicity by reducing the stress reaction in the endoplasmic reticulum and reducing the beta-cell apoptosis caused by glycolipid toxicity, thereby improving insulin secretion. They also proposed that the damaged function of the beta-cells will also be improved[19]. Nevertheless, the mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differentially abundant proteins in serum of T2DM patients have not been intensively explored so far.

Proteomics techniques have attracted more and more attention since these techniques can be used to identify the expression of differential proteins in cells and tissues of patients with T2DM[20]. Label-free quantitative proteomics can replace or supplement the traditional two-way gel electrophoresis approach, and it has become an important mass spectrometry method in recent years because of its powerful protein identification ability[21]. The changes in protein abundances of different samples can be analyzed by comparing mass spectrometry frequency or mass spectrometry peak intensity[22]. Without expensive isotope labeling and with liquid chromatography-mass spectrometry analysis of peptides obtained from protein enzymatic digestion, the relative abundance of the corresponding proteins can be quantified according to the signal strength of the peptide segments[22]. In this study, using this technique, we explored the differentially abundant proteins in the serum samples of T2DM patients before and after dapagliflozin treatments and conducted functional annotation analysis of the differential proteins. In addition, the levels of some differential proteins in serum samples were validated, and the correlations between their levels and clinical indexes were analyzed.

MATERIALS AND METHODS

Patients

Forty-six patients with T2DM were enrolled at the Department of Endocrinology, Affiliated Hospital of Inner Mongolia Medical University, between January 1, 2017 and December 1, 2018. There were 26 participants in the dapagliflozin group, and 20 participants who controlled their blood glucose levels through diet and exercise alone were enrolled in the control group during the same period (these patients did not receive drugs as per their own choice but were still followed in case diet and exercise became insufficient to control their T2DM). All participants met the diagnostic criteria for T2DM according to the World Health Organization diagnostic criteria for type 2 diabetes in 2017[23] The course of T2DM was < 5 years. All participants were 25-55 years of age, and they did not have a blood relationship. All participants had complete physical examination data and other disease information. The exclusion criteria were: (1) Acute or chronic complications of T2DM; (2) Cardiovascular and cerebrovascular diseases such as hypertension or coronary heart disease; (3) Other antidiabetic, antihypertensive, or lipid-regulating drugs; (4) Ketoacidosis, genital fungal infection, or urinary tract infection; (5) Recent history of surgery and trauma; or (6) Infectious diseases, tumors, hematological diseases, severe impairment of heart, liver and kidney functions, autoimmune diseases and other endocrine and metabolic diseases.

This study was approved by the Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University. All participants signed the informed consent form before the start of the study.

Study groups

For the participants in the dapagliflozin group, their HbA1c levels ranged from 7.0% to 10.0%. If the participants were taking other hypoglycemic drugs, the participants were admitted to the group after a wash-out period of 1-2 wk. The participants were instructed to adhere strictly to diet and exercise during the wash-out period. After wash-out, the participants were treated with dapagliflozin alone after clinical evaluation. The participants without the need for wash-out were treated with dapagliflozin directly after clinical evaluation. In order to avoid complications such as urinary tract infection and ketoacidosis, the participants were advised to drink more water during the study period, which was also conducive to the excretion of urinary glucose. No other drugs, such as lipid-lowering drugs, were allowed during the study. After 3 mo of treatment, the participants with HbA1c < 7.0% were considered as reaching the HbA1c target level. Six patients in the dapagliflozin group dropped out due to self-discontinuation, failure to meet the HbA1c target, or refusal to be reviewed after 3 mo. The average age of the 20 eligible patients in this group was 39.8 ± 5.1 years.

For the participants in the control group, their HbA1c levels reached the target level (HbA1c < 7.0%). These participants did not take any hypoglycemic drugs or other drugs within 3 mo before sampling, and they controlled their blood glucose levels only through diet and exercise. There were no dropouts. Their average age was 39.8 ± 6.0 years.

All participants received diet and exercise guidance. The dietary guidance referred to the balanced dietary plan recommended by the Chinese Guidelines for the Prevention and Treatment of Type 2 Diabetes (2013 edition) and suggested 1/3 structure of the energy intake ratio of three meals or 2:2:1 distribution. According to the "Guidelines for Exercise in Type 2 Diabetes Mellitus", the exercise program was mainly composed of low-intensity aerobic exercise such as walking, swimming, cycling,

etc. After a meal, the participants were required to exercise for about 30 min and 3-5 times per week. During the study, no adverse reactions such as urinary tract infection, ketoacidosis, or other adverse reactions occurred.

Data collection

Fasting venous blood was drawn from the participants in the morning. Blood samples were subjected to centrifugation. The supernatant was stored at -80 °C until analysis. Blood samples were also sent to the laboratory professionals of Affiliated Hospital of Inner Mongolia Medical University for quantification of indicators, including retinol-binding protein 4 (RBP4), fasting blood glucose (FBG), fasting C-peptide (FCP), fasting plasma insulin level (FINS), HbA1c, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol(LDL-C), triglycerides(TG), total cholesterol (TC), apolipoprotein A1 (ApoA1), ApoB100, homocysteine (HCY), non-esterified fatty acids (NEFA), C-reactive protein. These indicators were determined using Roche Cobas 8000 and Aikelai H-8160 automatic biochemical analyzers in the Laboratory Department of Affiliated Hospital of Inner Mongolia Medical University. Quality control was carried out by the professionals in the Laboratory Department. Insulin resistance indexes, including updated homeostasis model assessment-insulin resistance (HOMA2-IR), updated homeostasis model assessment-beta cell (HOMA2-B), and updated homeostasis model assessment-insulin sensitivity (HOMA2-S%), were obtained using the HOMA Calculator v2.2.3 software based on FPG, C-peptide, and islet function. The non-HDL-C value was calculated by subtracting the HDL-C value from the TC value.

Label-free proteomics

Serum samples from five patients in the dapagliflozin group before and after dapagliflozin treatments for 3 mo were selected for the exploratory proteomics study. Individual differences such as diabetes duration, blood glucose levels, and body mass index (BMI) were minimized to ensure the reliability of the results. High-abundance proteins were removed from the samples using Pierce™ Top 12 Abundant Protein Depletion Spin Columns Kit following the manufacturer's instruction, and total protein concentration was determined by a BCA kit (Pierce). The proteins were resolved by 10% SDS-PAGE and visualized with Coomassie Blue staining. Each lane was excised and cut into bands containing proteins with different molecular weights. Each gel fraction was subjected to in-gel tryptic digestion. Peptide segments were classified by high-pH reverse HPLC with Agilent 300 Extend C18 (5 μm diameter, 4.6 mm inner diameter, 250 mm long).

Digested peptides were analyzed by liquid chromatography-mass spectrometry (LC-MS)/MS using a ThermoScientific Easy nLC-1000 in tandem with a Q-Exactive Orbitrap mass spectrometer. Each sample (5 μL) was resolved using a 60 min gradient (Buffer A: 0.1 formic acid in 2% acetonitrile; Buffer B: 0.1% formic acid in 90% acetonitrile) on a 2 cm Acclaim 100 PepMap Nanoviper C18 trapping column in tandem with a Thermo EASY-Spray column (PepMap® RSLC, C18, 3 μm, 100 A, 75 μm × 150 mm).

Secondary mass spectrometry data were retrieved using Maxquant (v1.5.2.8).

The database was SwissProt Human (20387 sequences), which included the counter-library in calculating the false positive rate (FDR) caused by randomly matching. The common contamination library was considered to eliminate contaminations. The enzyme cutting method was set to trypsin/P; the number of leakage sites was set to 2. The first-stage mother ion mass error tolerance to the first search and main search was set to 20 ppm and 5 ppm. The error tolerance of the secondary fragment ions mass was set as 0.02 Da. The alkylation of cysteine was set as fixed modification, while the oxidation of methionine and acetylation of protein N-terminus were set as alternative modifications. The FDR of protein identification and PSM identification was set as 1%.

For data-dependent analysis, full scans were acquired at a 35000 resolution range of 400-200 m/z, while a 17500 resolution was used for MS/MS scans. Only the top 15 ions with +2 and +3 charges were selected for MS/MS with 10-s dynamic exclusion to prevent continuous reanalysis of abundant peptides. Following data acquisition, raw data files were compiled for each gel lane and searched with Proteome Discoverer 1.4's SEQUEST search algorithm using the reviewed, non-redundant homo sapiens complete proteome retrieved from UniProtKB.

Bioinformatics analysis

For protein functional enrichment evaluation, Gene Ontology (GO) and pathway enrichment analyses were carried out. GO annotations (including biological process (BP), molecular function (MF), and cellular component) were performed using the InterProScan database (v.5.14–53.0 http://www.ebi.ac.uk/interpro/) and according to an existing report[16]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway enrichment analysis[16]. KAAS (v.2.0 http://www.genome.jp/kaas-bin/kaas_main) and KEGG mapper (v.2.5 http://www.kegg.jp/kegg/mapper.html) are the main tools used with the KEGG database. Prediction of subcellular localization was carried out using the wolfpsort software (v.0.2 http://www.genscript.com/psort/wolf_psort.html). Cluster memberships were visualized using the heat map drawn by the function heatmap.2 in the R package gplots. For each annotation, Fisher's exact test was applied to

compare the enrichment of the differentially abundant protein against all identified proteins, and a P value < 0.05 was considered significant.

Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) was performed for the quantification of alpha II B integrin, myeloperoxidase (MPO), and podocalyxin (PCX) using the kits from Bio-Rad Laboratories, United States (CSB-EL0118644HU for integrin, CSB-E08721h for MPO, and CSB-E09891h for PCX). All reagents were equilibrated to room temperature (18-25 °C) for at least 30 min and prepared according to the instructions of the relevant kits. The optical density of each well was measured sequentially at 450 nm with an enzyme-labeled instrument within 5 min after the termination of the reaction.

Statistical analysis

The statistical methods of this study were reviewed by Xue-Mei Wang. The quantitative data were described using means ± SD. The paired *t*-test was used to compare the indexes obeying the normal distribution before and after treatment. Variance analysis was used to compare the indexes obeying normal distribution, and a nonparametric rank-sum test was used to compare the indexes not obeying normal distribution. Pearson correlation analysis was used for the two variables obeying normal distribution, and Spearman grade correlation analysis was used for the variables not obeying normal distribution. The chi-square test was used to analyze the categorical data. For the enrichment test, Fisher's exact test was used to show the functional classification and pathway of significant enrichment of differentially expressed proteins (P < 0.05) using bubble diagrams. Two-sided P < 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS 22.0.

RESULTS

Dapagliflozin significantly improved the clinical manifestations in patients with T2DM

Twenty participants were treated from January 1, 2017 to December 1, 2018. Table 1 presents the characteristics of the participants in the dapagliflozin (after treatment) and control groups. Compared with the controls, the patients under dapagliflozin treatment had slightly higher FBP, lower FINS, and lower HOMA2-B, but no significant differences in HOMA2-S% and HOMA2-IR. Compared with the controls, patients with dapagliflozin had higher apoA1 Levels and lower NEFA levels. Table 2 presents the comparison before/after dapagliflozin. Compared with baseline, the participants after dapagliflozin treatment had significantly decreased BMI, waist circumference, and waist-hip ratio (P < 0.05) (Table 2). In addition, their blood glucose-related indexes, including HbA1c, FCP, FINS, FBP, and HOMA2-IR, also demonstrated significantly decreased levels (P < 0.05), while HOMA2-S% and HOMA2-B significantly increased (P < 0.05) (Table 2). Regarding the lipid metabolism-related indexes, non-HDL-C decreased (P < 0.05), while ApoA1 increased (P < 0.05) (Table 2). Moreover, RBP, HCY, and NEFA were significantly decreased levels after treatment (P < 0.05) (Table 2). Taken together, dapagliflozin substantially improved the clinical manifestation of patients with T2DM.

Identification of differentially abundant proteins by label-free proteomic

Next, we performed a label-free proteomics analysis of serum samples from five patients before and after dapagliflozin treatments. A total of 665007 sary spectra were obtained through mass spectrometry. A total of 4732 peptides were identified through spectral analysis, of which 3389 were specific peptides. We identified 534 proteins, all of which could be quantified (quantitative protein means that at least one comparison group has quantitative information). The evaluation of quantitative proteome reproducibility was performed by relative standard deviation (RSD) analysis, which showed that the biological replicates were statistically consistent (Figure 1A). The heatmap of the Pearson correlation coefficients from all quantified proteins between each pair of samples demonstrated that the linear correlation degree of the two metrics was not high (Figure 1B). Notably, 19 proteins exhibited significant differences before and after dapagliflozin treatments (P < 0.05; FC > 1.5), of which 18 were downregulated, and one was upregulated (Figure 1C).

In order to determine the characteristics of the differentially expressed proteins, we annotated the subcellular localization, Clusters of Orthologous Groups of proteins (KOG), Gene Ontology (GO), and KEGG pathway of the 19 differentially expressed proteins. The annotation of the subcellular localization showed that 31.6% of all differentially expressed proteins were localized to the cytoplasm, 26.3% to the extracellular space, 15.8% to the nucleus, 10.5% to the plasma membrane, 5.3% to the mitochondria, 5.3% to the endoplasmic reticulum, and 3.8% to the cytoskeleton (Figure 2A). In the GO analysis, the GO annotations are divided into three categories (biological process, cellular component, and molecular function), explaining the biological role of proteins from different perspectives. In the biological process classification of GO, the cellular process had the largest proportion, followed by the single-organism process and biological regulation. In the cellular composition classification, differentially expressed proteins were mostly expressed in organelles, followed by cells and extracellular regions. In the

Table 1 Analysis of clinical indexes between the dapagliflozin and control groups in patients with type 2 diabetes mellitus (means ± SD, *n* = 20)

Parameter	Dapagliflozin	Control	Wilcoxon W	*P* value
Age	39.80 ± 5.136	39.75 ± 5.990	397.000	0.724
Smoking history	16.55 ± 9.720	10.25 ±11.639	353.000	0.111
Height (m)	1.7670 ± 0.07255	1.7505 ± 0.090	389.000	0.569
Weight (kg)	74.950 ± 8.9176	72.525 ± 6.6718	381.500	0.440
BMI (kg/m^2)	24.3695 ± 1.82674	24.014 ± 1.6715	385.00	0.499
Waist circumference (cm)	96.350 ± 2.3402	93.525 ± 5.7502	325.000	0.021
Hip circumference (cm)	96.975 ± 2.4413	99.225 ± 6.4062	398.500	0.755
Waist-hip ratio	0.9937 ± 0.0132391	0.9494 ± 0.04815	308.000	0.006
SBP (mmHg)	124.80 ± 5.297	120.40 ± 5.423	323.500	0.018
DBP (mmHg)	78.85 ± 5.724	77.95 ± 5.969	394.500	0.671
Diabetes course (yr)	2.575 ± 1.2169	2.825 ± 1.8229	396.500	0.713
TG	1.884 ± 1.2172	0.979 ± 0.2376	1.2172203	0.002
LDL	2.8585 ± 1.0440	1.5975 ± 0.5079	1.0439867	< 0.001
HDL	1.0855 ± 0.2457	1.023 ± 0.1246	0.2457100	0.560
APOA1	1.3630 ± 0.2022	1.2625 ± 0.1882	0.2022271	0.010
APOB100	0.9425 ± 0.2651	1.0345 ± 0.1521	0.2651092	0.424
CRP	1.311 ± 0.9305	1.1635 ± 0.5462	0.9305511	0.967
HbA1c	6.7500 ± 0.6863	6.3700 ± 0.3827	0.6863327	0.032
THCY	12.030 ± 2.1451	12.4215 ± 1.2702	2.1450899	0.213
RBP4	37.150 ± 5.6033	35.800 ± 4.3842	5.6033	0.446
NEFA	0.8245 ± 0.5849	1.026 ± 0.1099	0.5849019	< 0.001
FCP	2.2770 ± 0.7903	2.231 ± 0.4151	0.7903104	0.525
TC	4.1950 ± 0.8378	3.7875 ± 0.4683	344.500	0.076
IR	2.7120 ± 1.7245	2.8440 ± .8424	358.500	0.163
FINS	8.4905 ± 4.4824	10.313 ± 3.2099	328.500	0.027
FBP	6.80 ± 1.105	6.15 ± 0.671	335.500	0.034
Non-HDL	3.05 ± 0.826	2.80 ± 0.410	364.000	0.142
HOMA2- insulin	75.6150 ± 20.339	89.460 ± 18.246	324.000	0.020
HOMA2-S%	61.6750 ± 21.1603	60.440 ± 14.789	400.000	0.787
HOMA2-IR	1.8185 ± 0.7285	1.7210 ± 0.3760	399.000	0.766

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglycerides; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; APOA1: Apolipoprotein A1; ApoB100: Apolipoprotein B100; CRP: C-reactive protein; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; THCY: Homocysteine; RBP4: Retinol binding protein 4; NEFA: Non-esterified fatty acids; FCP: Fasting C-peptide; TC: Total cholesterol; FINS: Fasting plasma insulin level; HOMA2-B: Homeostatic model assessment-beta cell; HOMA2-S%: Homeostatic model assessment-insulin sensitivity; HOMA2-IR: Homeostatic model assessment-insulin resistance.

molecular function classification, binding molecules accounted for the largest proportion (Figure 2B). In terms of subcellular structure location, these differentially expressed proteins were mainly located in the cytoplasm, extracellular, nucleus, and plasma membrane. COG/KOG functional classification revealed that most of these differentially expressed proteins played a role in the cytoskeleton, extracellular structures, intracellular trafficking, secretion, vesicular transport, carbohydrate transport, and metabolism, as general function predictions (Figure 2C).

Table 2 Analysis of clinical indexes before and after dapagliflozin treatment in patients with type 2 diabetes mellitus (means ± SD, *n* = 20)

Parameter	Before dapagliflozin	After dapagliflozin	Wilcoxon W	*P* value
Weight (kg)	80.600 ± 10.4549	74.950 ± 8.9176	351.000	0.110
BMI	26.1875 ± 2.1889	24.3695 ± 1.8267	313.000	0.009
Waist circumference (cm)	98.300 ± 2.4570	96.350 ± 2.3402	310.50	0.007
Hip circumference (cm)	97.425 ± 3.1131	96.975 ± 2.4413	395.000	0.684
Waist-hip ratio	1.009350 ± 0.0172	0.9937 ± 0.0132	311.500	0.007
TG	2.3020 ± 1.7483	1.884 ± 1.2172	387.000	0.534
LDL	2.9310 ± 0.9873	2.8585 ± 1.0440	405.000	0.892
HDL	1.0525 ± 0.2722	1.0855 ± 0.2457	381.000	0.432
APOA1	1.2450 ± 0.2176	1.3630 ± 0.2022	334.500	0.041
APOB100	1.0345 ± 0.3400	0.9425 ± 0.2651	392.000	0.626
CRP	1.7090 ± 1.1454	1.311 ± 0.9305	349.000	0.098
HbA1c	8.0550 ± 1.0842	6.7500 ± 0.6863	277.000	< 0.001
THCY	14.6850 ± 3.0387	12.030 ± 2.1451	311.500	0.008
RBP4	43.310 ± 8.8547	37.150 ± 5.6033	315.500	0.010
NEFA	0.4525 ± 0.3246	0.8245 ± 0.5849	321.500	0.017
FCP	2.7980 ± 0.9510	2.2770 ± 0.7903	337.000	0.048
TC	4.6830 ± 1.1643	4.1950 ± 0.8378	347.500	0.091
IR	5.7385 ± 3.2238	2.7120 ± 1.7245	266.000	< 0.001
FINS	13.3500 ± 5.7057	8.4905 ± 4.4824	289.000	0.001
FBP	9.60 ± 2.437	6.80 ± 1.105	245.000	< 0.001
Non-HDL	3.70 ± 1.129	3.05 ± 0.826	337.000	0.037
HOMA2-insulin	53.4450 ± 21.1716	75.6150 ± 20.339	305.000	0.005
HOMA2-S%	44.7950 ± 16.2213	61.6750 ± 21.1603	310.500	0.007
HOMA2-IR	2.5185 ± 0.9686	1.8185 ± 0.7285	313.000	0.009

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglycerides; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; APOA1: Apolipoprotein A1; ApoB100: Apolipoprotein B100; CRP: C-reactive protein; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; THCY: Homocysteine; RBP4: Retinol binding protein 4; NEFA: Non-esterified fatty acids; FCP: Fasting C-peptide; TC: Total cholesterol; FINS: Fasting plasma insulin level; HOMA2-B: Homeostatic model assessment-beta cell; HOMA2-S%: Homeostatic model assessment-insulin sensitivity; HOMA2-IR: Homeostatic model assessment-insulin resistance.

Functional enrichment analysis of differentially abundant proteins

For the annotation of all identified proteins and the screening of differentially expressed proteins, the differentially expressed proteins in our comparison groups were enriched at three levels: GO classification, KEGG pathway, and protein domains. The purpose was to determine whether the differentially expressed proteins had significant enrichment trends in some functional types. GO functional classification found that the homotypic intercellular adhesion pathway, lymphocyte activation pathway, and actin cytoskeleton regulation pathway were highly enriched in the classification of cell composition (Figure 3A). The pathways such as Cortex and Cytoskeleton were enriched significantly in the KEGG pathway (Figure 3B). In the function of molecular biology, the small GTP enzyme binding pathway and the Ras GTPase binding pathway were enriched significantly (Figure 3C).

We found that these proteins, such as actin, alpha II beta integrin, MPO, and PCX, were closely related by the KEGG pathway. Therefore, we performed a protein-protein interaction analysis and identified a network among the integrin protein, MPO, and PCX (Figure 4). The integrin protein was used to modulate the production of cytoplasmic actin by participating in the PKC and the FAK pathway to function on actin filament and vinculin. On the other hand, by participating in the PKC and FAK pathway to function on Rac family proteins, the integrin protein modulates the MAPK signal through the PAK pathway to eventually manipulate gene expression. Besides, the integrin protein can also act

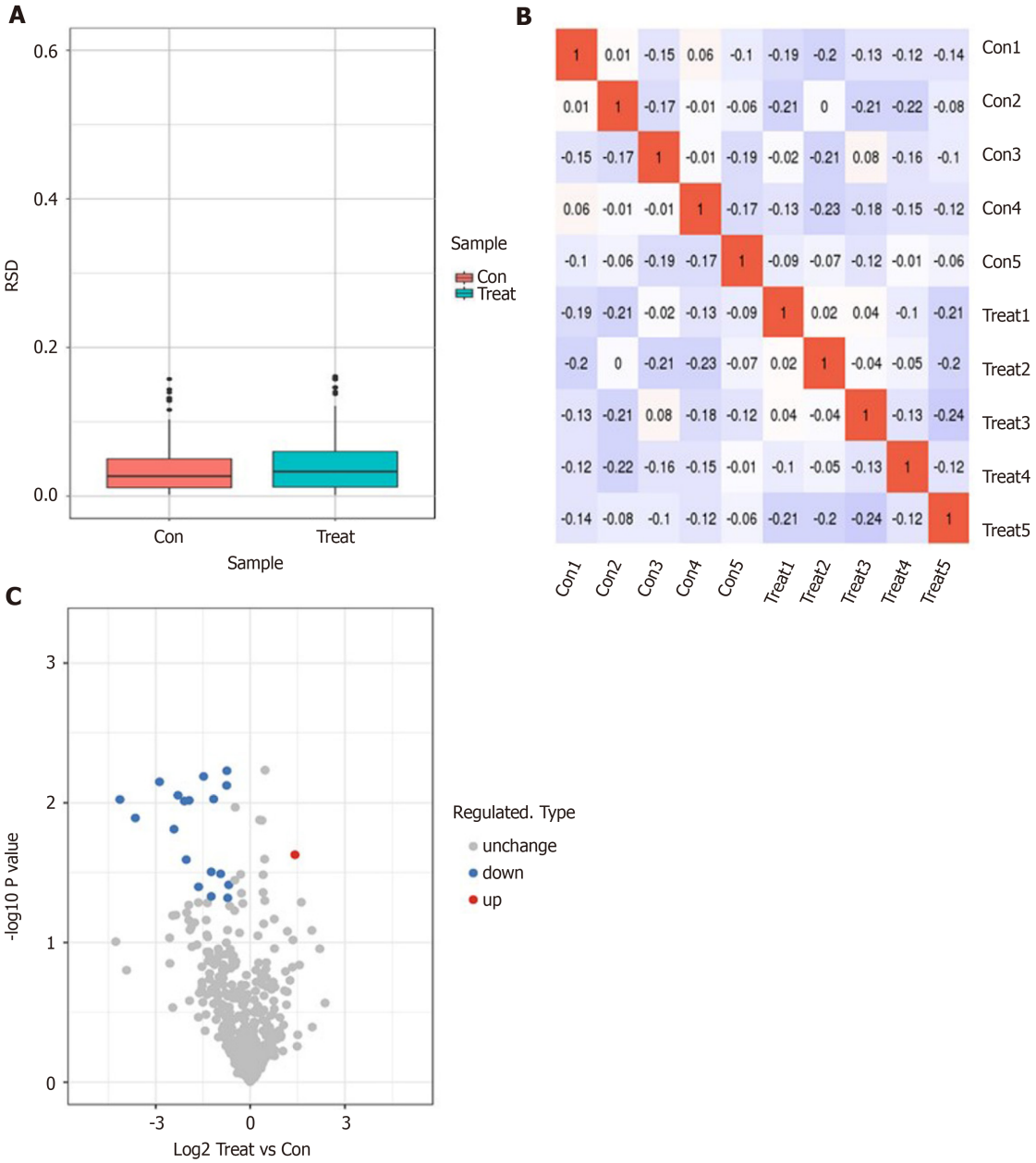

Figure 1 Identification of dapagliflozin treatment-associated differentially abundant proteins in serum samples from type 2 diabetic patients by label-free proteomics. A: Box plot of relative standard deviation (RSD) distribution of repeated samples using quantified proteins. A box plot drawn by the RSD of the quantitative protein value between replicate samples is shown. The smaller the overall RSD value is, the better the quantitative repeatability is; B: Heatmap of Pearson correlation coefficients from all quantified proteins between each pair of samples is shown; C: The volcano plot demonstrated differentially expressed proteins. The horizontal axis is the relative quantitative value of the protein after Log2 Logarithmic conversion, and the vertical axis is the value of the difference significance test *P* value after -Log10 Logarithmic conversion. The red dots in the figure indicate proteins with significantly differentially upregulated expression, and blue dots indicate proteins with significantly differentially down-regulated expression.

on the PI3K pathway through the SRC family, affecting Notch signaling pathway. The cytoplasmic actin is involved in the behavior of phagolysosome with coronin, and the MPO production is increased during the formation of autophagolysosome. PCX protein and actin are involved in regulating the actin skeleton, and the Notch signaling pathway also participates in this process (Figure 4).

Validation of three differentially expressed proteins and their correlations with clinical indexes

Based on the literature and our analysis of the biological function of candidate proteins that might be closely related to diabetes mellitus, three differentially expressed proteins (including MPO, alpha II B integrin, and PCX) were validated by ELISA. The results showed that the expression of MPO and alpha II B integrin protein in serum samples was significantly downregulated ($P < 0.05$), and the PCX protein levels were significantly upregulated ($P < 0.05$) after dapagliflozin treatment (Figure 5A). In addition, compared with the control group, the dapagliflozin group also had a significantly downregulated expression of MPO after treatment ($P < 0.05$) (Supplementary Figure 1). We also conducted a correlation

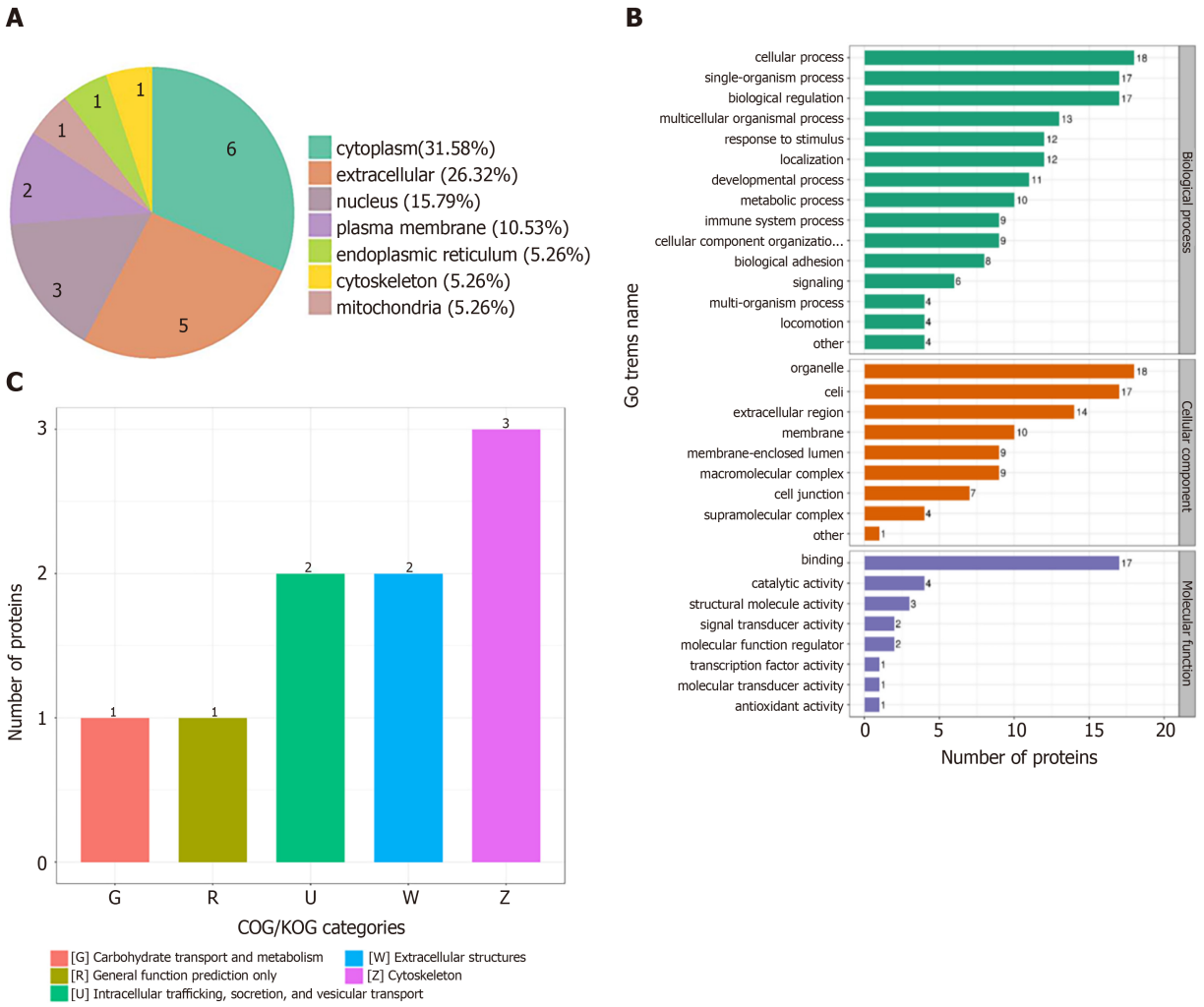

Figure 2 Annotation analysis of 19 differentially expressed proteins by subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations. A: Subcellular localization chart of differentially expressed proteins; B: Statistical distribution chart of differentially expressed proteins under each Gene Ontology category (2nd Level); C: EuKaryotic Orthologous Groups functional classification chart of differentially expressed proteins.

analysis between the expression levels of these three differentially expressed proteins and clinical indexes (Supplementary Table 1). We found that the serum alpha II B integrin protein levels were positively correlated with THCY (homocysteine) and FCP ($P < 0.05$, Figure 5B), while that of PCX was positively correlated with HOMA2-B and negatively correlated with HbAc1 and FBP ($P < 0.05$, Figure 5C). In addition, the serum protein levels of MCP were positively correlated with multiple parameters, including HbAc1, RBP, FCP, FINS, N-HDL, and HOMA2-IR, and negatively correlated with HOMA2-S% ($P < 0.05$, Figure 5D).

DISCUSSION

The SGLT-2 inhibitor dapagliflozin, as a new hypoglycemic drug, plays a role in lowering blood glucose by reducing the reabsorption of SGLT2 receptor glucose in renal tubular epithelial cells in patients with T2DM. Here, we performed label-free quantitative proteomics analysis of serum samples in patients before and after dapagliflozin treatments and identified differentially expressed proteins associated with dapagliflozin treatment. Notably, our function annotation and enrichment analysis suggested that three differential proteins (including αIIβ integrin, MPO, and PCX) potentially contribute to the renal and cardiovascular protective roles of dapagliflozin through participating in the regulation of multiple pathways. Furthermore, the serum differential expressions of these proteins were validated by ELISA, and their levels were correlated with some clinical indexes in patients with T2DM.

This study used dapagliflozin as the representative drug for SGLT-2 inhibitors. We found that the related indexes of islet function, such as FBG, HbA1c, FCP, FINS, and HOMA2-IR, decreased after dapagliflozin treatment. The lipid metabolism of T2DM patients was significantly improved after dapagliflozin treatment, as evidenced particularly by decreased non-HDL-C and increased ApoA1

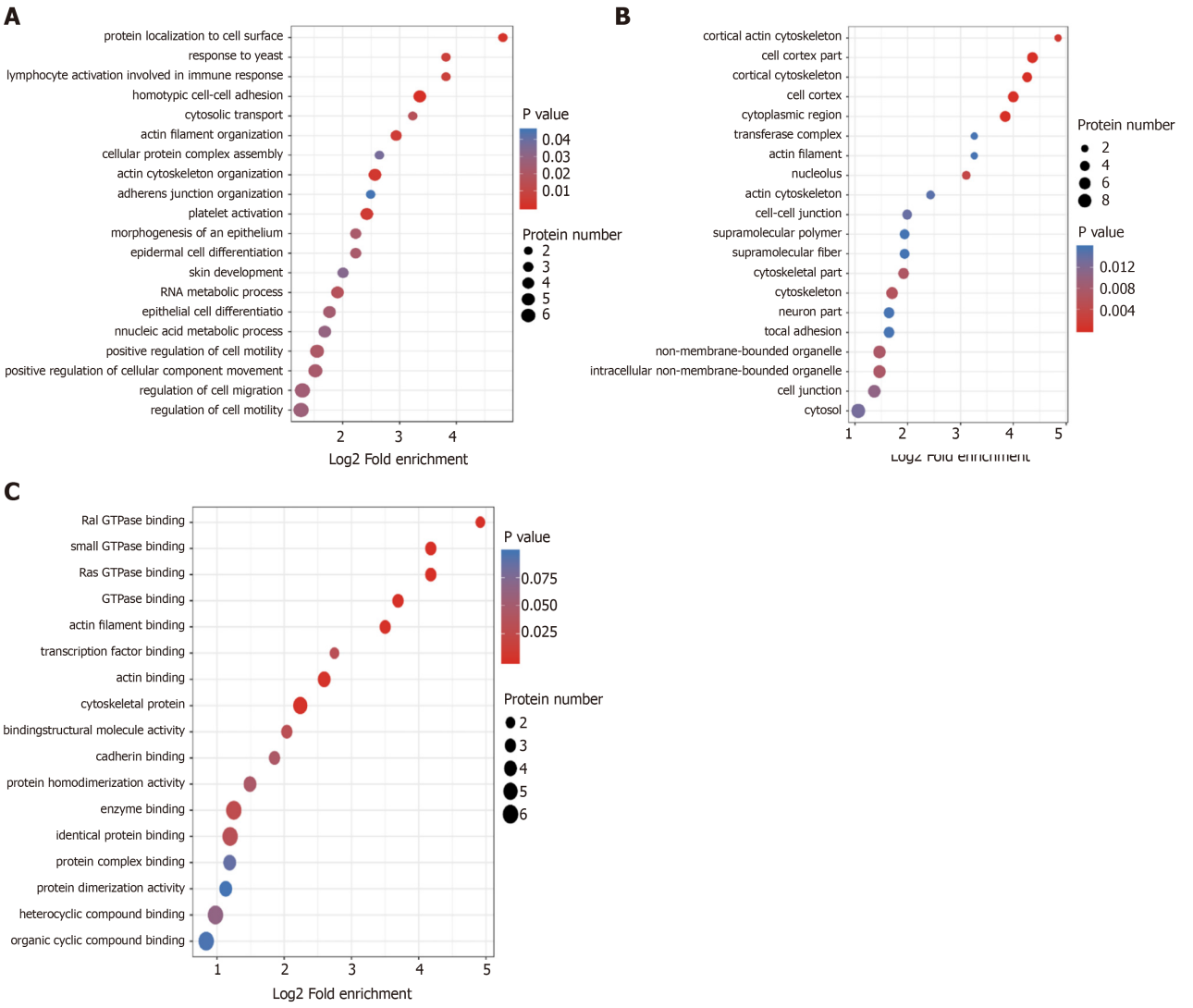

Figure 3 Gene Ontology enrichment bubble plots of differentially expressed proteins in three categories. A-C: The Gene Ontology enrichment bubble plots in the categories of biological process (A), cellular component (B), and molecular function (C) are shown. The bubble chart shows the results of the top 20 categories with the most significant enrichment. In the bubble chart, the vertical axis is the function classification or pathway, and the horizontal axis is the value after Log2 conversion of the ratio of the differential protein in the functional type compared to the ratio of the identified protein. The circle's color indicates the *P* value of enrichment significance, and the size of the circle indicates the number of differential proteins in the functional class or pathway.

Levels. Compared with baseline, the participants after dapagliflozin administration had significantly decreased BMI and hip circumference. We also found that RBP4, NEFA, and HCY were decreased after treatment with dapagliflozin. The controls were patients with mild T2DM in whom diet and exercise were sufficient to control their condition. Of note, dapagliflozin decreased FBG from 9.60 ± 2.44 to 6.80 ± 1.11 mmol/L, close to the value in controls (6.15 ± 0.67 mmol/L). Dapagliflozin also decreased FINS to lower levels than in controls, but HOMA2-B was lower than in controls, while there were no significant differences in HOMA2-S% and HOMA2-IR between the two groups. Compared with the controls, patients with dapagliflozin had higher apoA1 Levels and lower NEFA levels, also supporting the benefits of dapagliflozin. These results support the known effects of dapagliflozin in patients with T2DM[15]. RBP4, as a fat-derived factor, is closely related to obesity, insulin resistance, and other diseases[24]. Ost *et al*[25] found that RBP4 can prevent insulin-stimulated serine phosphorylation at position 307 of IRS1 by interfering with RBP4 and its antibodies in primitive adipocytes and correspondingly increasing the effective concentration of IRS1 tyrosine phosphorylation by half, as well as preventing the phosphorylation of ERK1/2. Therefore, it can be concluded that RBP4 might interfere with the Ras/MAPK signal of the insulin receptor by interfering with the phosphorylation of ERK1/2, thus participating in insulin resistance. In this study, after label-free quantitative proteomics, we also found that the differentially expressed proteins were significantly enriched in the Ras GTPase pathway. Since these two metabolic pathways share a common pathway, whether dapagliflozin could play a role through this pathway and the mechanism of RBP4 in dapagliflozin effectiveness need to be further studied.

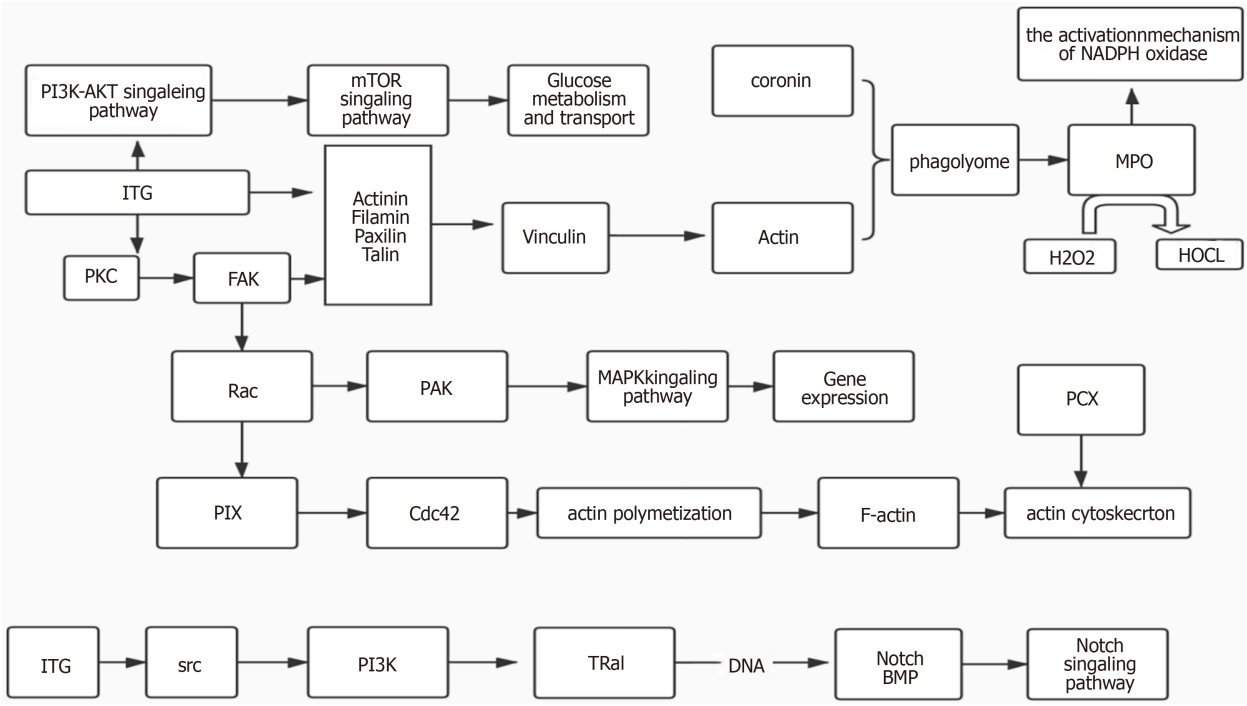

Figure 4 Treemap chart shows the interaction among differentially expressed proteins. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis identified a protein-protein interaction network among the integrin protein, myeloperoxidase, and podocalyxin.

Our comparative analysis with non-standard quantification demonstrated that 18 proteins were downregulated, and one was upregulated in serum samples of patients after dapagliflozin treatment. After consulting the literature, three candidate differential proteins closely related to T2DM were selected for ELISA validation. The differential expressions of these proteins were validated using serum samples from all the patients before and after dapagliflozin treatments. Notably, the correlation analysis suggested that the serum MPO protein levels were positively correlated with multiple clinical indexes, and the alpha II B protein levels were positively correlated with THCY and FCP levels. In addition, the serum PCX levels were negatively correlated with HOMA2-B and positively correlated with HbA1c and FBP. Based on these results and the literature, these three differential proteins might contribute to the beneficial roles of dapagliflozin in treating T2DM patients. Of note, PCX is the main surface antigen of podocytes and is normally expressed in renal podocytes, endothelial cells, and vascular endothelial cells and participates in maintaining the vascular endothelial cell barrier and reducing vascular inflammation. High glucose levels downregulate the expression of PCX in cultured podocytes *via* ERK1/2 MAPKs and inhibit the expression of PCX protein and mRNA by WT1 tumor protein and advanced glycation end-products[26], possibly resulting in the reduction of PCX in the blood. Second, we agree that integrins are cellular proteins that would not be expected to be found in circulation. Still, this study was not designed to determine the source of these proteins in the plasma. On the other hand, numerous studies report serum/plasma levels of various integrins as markers of diseases[27-29]. It could be hypothesized that the systemic inflammatory condition observed in T2DM increases cell death, releasing those proteins in circulation, but the present study cannot provide an answer regarding that point. Future studies will have to examine that specifically.

MPO is a heme enzyme and is the major protein in neutrophils and, to a lesser extent, in monocytes. MPO uses H_2O_2 to generate HOCl, a potent bactericidal agent, generating ROS[30]. MPO plays an essential part in the innate immune system by catalyzing the production of HOCl[31], but MPO has also been implicated as a very harmful agent in an increasing number of inflammatory-mediated disorders [32]. It has been reported that MPO is related to insulin resistance and inflammation parameters in overweight subjects with first-degree relatives of T2DM[31]. In addition, plasma MPO levels were positively correlated with the degree of coronary artery stenosis in T2DM patients, and increasing blood glucose might amplify the association between MPO and coronary artery disease[33]. Patients with uremic diabetic nephropathy with a low MPO level might be at a lower risk for any cardiac event than uremic patients with high MPO levels, suggesting that MPO might be a biomarker to predict coronary events in diabetic patients end-stage renal disease[34]. In this study, MPO levels in the dapagliflozin group decreased after treatment, related to the improvement of blood glucose control and inflammatory oxidation. Since our correlation analysis suggested that MPO was positively correlated with indexes including RBP, FCP, FINS, N-HDL, and HOMA2-IR, MPO very likely participated in oxidative stress regulation and acute, chronic inflammation of T2DM through a certain metabolic pathway. However, the specific mechanisms of MPO in contributing to the beneficial roles of dapagliflozin are still under

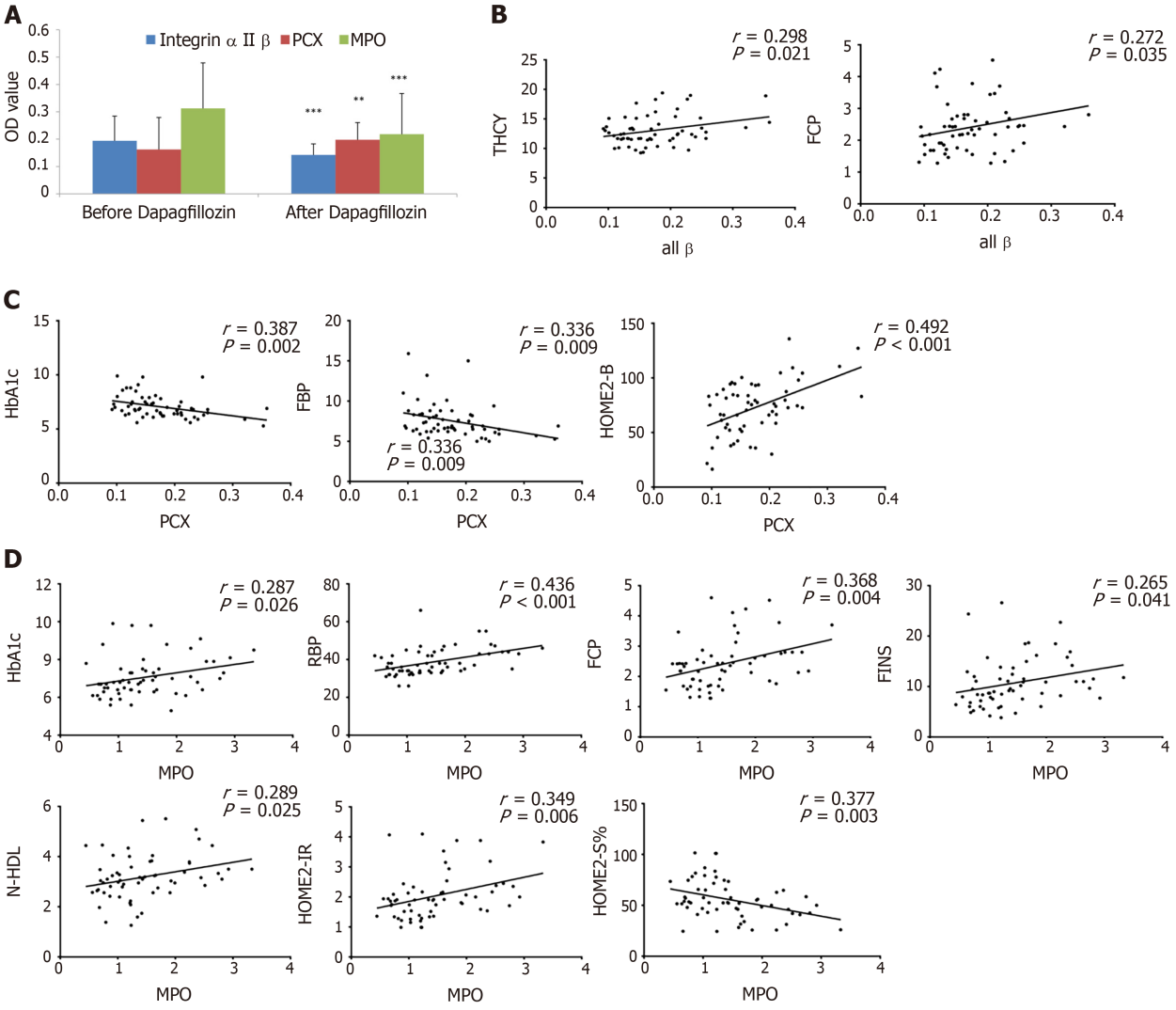

Figure 5 Correlation analyses of the expression levels of three differentially expressed proteins and clinical indexes of type 2 diabetic patients. A: The levels of three differentially abundant proteins, including myeloperoxidase (MPO), alpha II B integrin, and podocalyxin (PCX) proteins in serum samples of patients before and after dapagliflozin treatments were evaluated by enzyme linked immunosorbent assay. $n = 20$ for each group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, between the indicated groups; B-D: Dot plots show the Pearson correlations between the levels of alpha II B integrin (B), PCX (C), and MPO (D) proteins and some clinical indexes in patients before and after dapagliflozin treatments.

investigation.

Integrin is a transmembrane protein that exists on the cell membrane to mediate cell-to-cell interaction. It can interact with various growth factors at the receptor level and regulate cell adhesion, survival, growth, differentiation, proliferation, and migration[35]. There are 24 kinds of integrins in humans, formed by heterodimerization of 18 alpha subunits and eight beta subunits. Alpha II beta integrin is one of the important components of the integrin family[35]. Studies have confirmed that platelet apoptosis in patients with T2DM is higher than in normal patients, increasing alpha II beta integrin levels[36]. The mechanism might be that integrins participate in RAS/MAPK signal transduction through interaction with growth factors and synergism between integrins, related to insulin resistance and secretion deficiency[37,38]. It has not been reported through which pathway dapagliflozin can induce the downregulation of alpha II beta integrin. Our analysis indicated that the Ras GTP pathway enrichment might be related to the downregulation of alpha II beta integrin in the differential protein function enrichment pathway in this study, which needs further confirmation by additional experiments.

The normal expression of PCX can prevent negatively charged proteins from leaking into human urine, resist adhesion between adjacent foot processes, and prevent adhesion between parietal epithelial cells and capillary loops[39]. Through animal experiments, Qi *et al*[40] demonstrated that high glucose could activate the ERK1/2 MAPK pathway of podocytes and decrease PCX expression. Although hyperglycemia inhibits PCX expression during glomerular injury, it can aggravate podocyte injury, increasing PCX with urine excretion[40]. These results suggest that urinary PCX is increased in urinary nephropathy, but PCX expression decreases with high glucose levels. We found that PCX was

negatively correlated with HbA1c and FBP. The lipid metabolism disorders in diabetic nephropathy were higher than in diabetic patients without nephropathy, and there were obvious disorders in the early stage of diabetic nephropathy (Figure 5B). This study suggests that dapagliflozin might participate in the protective effect of PCX protein in kidney through lipid metabolism-related mechanisms, and there are many pieces of evidence. Dapagliflozin has a protective effect on kidney by affecting glomerular feedback, restoring renal blood flow and glomerular filtration rate, thus preventing the progression of diabetic nephropathy in the initial stage, and posing potential renal protective effect on patients with mild to moderate renal insufficiency[13-15]. SGLT2 inhibitors can also limit the glycotoxicity of the kidney itself and reduce renal hypertrophy[13-15]. Vallon *et al*[41] suggested that SGLT2 inhibitors could inhibit the expression of inflammatory markers and fibrotic markers, and whether PCX was involved in these processes needs further study.

This study has several limitations. Because it was an exploratory study, no power analysis was initially performed, and the participants were enrolled using convenience sampling. Five serum samples with relatively small heterogeneity were directly selected from the dapagliflozin group for LC-MS. The abundance of proteins in the different samples was analyzed by comparing the frequency of mass spectrometry analysis or the peak intensity of mass spectrometry. Because a single sample can only be analyzed separately, the analytical flux was relatively low, and repeated experiments are needed to improve the accuracy of the analytical flux. Moreover, the sample size of this study is insufficient. Due to limited time and funding, the mechanisms of the differentially expressed proteins associated with dapagliflozin treatments need to be further studied. Many proteins' bioinformatics database data collection might be needed for analyzing the interaction between identified differentially expressed proteins and predicting the correlation between these differentially expressed proteins and clinical indicators. In addition, the relationship between dapagliflozin treatments and the changes of isoproteins still needs to be further validated.

CONCLUSION

Dapagliflozin has obvious hypoglycemic effects, and it can also improve weight loss, lipid metabolism, and islet function of patients with T2DM. After dapagliflozin treatment, 18 proteins (including MPO and alpha II beta integrin) were downregulated, and PCX protein was upregulated in the serum of T2DM patients. Subsequent function annotation and enrichment analysis, as well as ELISA validation and correlation analysis with the clinical indexes, suggested that MPO, alpha II beta integrin, and PCX might contribute to the beneficial roles of dapagliflozin through their regulations on oxidative stress, insulin resistance, and lipid metabolism.

ARTICLE HIGHLIGHTS

Research background

Only 50% of patients with type 2 diabetes mellitus (T2DM) can control their blood glucose levels. Dapagliflozin is a selective inhibitor of sodium-glucose co-transporter 2 (SGLT-2) that improves the insulin sensitivity of the liver and peripheral tissues. Many studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection.

Research motivation

The mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differentially expressed proteins in the serum of T2DM patients have not been intensively explored so far.

Research objectives

This study aimed to identify differentially expressed proteins associated with dapagliflozin treatment in patients with T2DM. The results could help understand the mechanisms of dapagliflozin in patients with T2DM.

Research methods

Twenty T2DM patients [hemoglobin A1c (HbA1c) 7.0%-10.0%] were enrolled at The Affiliated Hospital of Inner Mongolia Medical University between January 1, 2017 and December 1, 2018. They received dapagliflozin (10 mg/d) for 3 mo, and the HbA1c < 7.0% target was achieved. The changes in clinical indexes were compared before and after treatments. Label-free quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. The identified differentially expressed proteins were analyzed using various bioinformatics tools.

Research results

Dapagliflozin significantly improved the clinical manifestation of the patients. There were 18 downregulated proteins and one upregulated protein in the serum samples of patients after dapagliflozin administration. Bioinformatics analyses, including subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations, were used to profile the biological characteristics of the 19 differentially expressed proteins. Based on the literature and function enrichment analysis, two downregulated proteins, myeloperoxidase (MPO) and alpha II B integrin, and one upregulated protein, podocalyxin (PCX), were selected for enzyme linked immunosorbent assay (ELISA) validation. These validated differentially expressed proteins had multiple correlations with clinical indexes, including HbAc1 and fasting C-peptide.

Research conclusions

Dapagliflozin has obvious hypoglycemic effects, and it can also improve weight loss, lipid metabolism, and islet function of patients with T2DM. After dapagliflozin treatment, 18 proteins (including MPO and alpha II beta integrin) were downregulated, and PCX protein was upregulated in the serum of T2DM patients.

Research perspectives

Subsequent function annotation and enrichment analysis, as well as ELISA validation and correlation analysis with the clinical indexes, suggested that MPO, alpha II beta integrin, and PCX might contribute to the beneficial roles of dapagliflozin through their regulations on oxidative stress, insulin resistance, and lipid metabolism.

FOOTNOTES

Author contributions: Zhao YX contributed to methodology, software, formal analysis, investigation, resources, data curation, writing original draft preparation, writing review and editing; Borjigin S contributed to experimental operation. Yan ZL contributed to conceptualization, methodology, validation, formal analysis, investigation, resources, writing original draft preparation, writing review and editing, supervision, funding acquisition; all authors have read and agreed to the published version of the manuscript.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Higher risk of type 2 diabetes in young women with polycystic ovary syndrome: A 10-year retrospective cohort study

Wan-Ting Liao, Jing-Yang Huang, Ming-Tsung Lee, Yu-Cih Yang, Chun-Chi Wu

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Abstract

BACKGROUND

Polycystic ovary syndrome (PCOS) is a common disorder in women of reproductive age. Over the last few decades, research studies have revealed that PCOS is strongly associated with metabolic disorders, including metabolic syndrome, obesity, insulin resistance and prediabetes. Clinical observation has shown that women with PCOS are expected to have an increased risk of developing type 2 diabetes (T2DM) in the future.

AIM

To assess the hazard ratio (HR) of T2DM between women with/without PCOS.

METHODS

This population-based, retrospective cohort study evaluated data retrieved from the National Health Insurance Research Database. The subjects were women with PCOS (n = 2545) identified on the basis of diagnosis, testing, or treatment codes, and women without PCOS as controls (n = 2545). The HR of T2DM between women with or without PCOS was the main outcome measure analyzed.

RESULTS

Our study found that, during a 10-year follow-up period, the overall incidence of T2DM was 6.25 per 1000 person-years in the PCOS group compared with 1.49 in the control group. After adjustment for potential confounding variables, the overall incidence of T2DM was higher in the PCOS group *vs* the control group (HR = 5.13, 95%CI: 3.51-7.48, $P < 0.0001$). The risk of developing T2DM subsequent to PCOS decreased with increasing diagnosis age: the adjusted HR was 10.4 in the 18–24-year age group, 5.28 in the 25-29-year age group, and 4.06 in the 29-34-year age group. However, no such significant association was noted in women older than 35 years.

CONCLUSION
These findings highlight the importance of prompting a more aggressive treatment to prevent diabetes in women diagnosed with PCOS at a young age, and, in contrast, the lessened importance of this type of intervention in women diagnosed with PCOS at a late reproductive age.

Key Words: Polycystic ovary syndrome; Diabetes; Incidence; Hazard ratio; Population-based cohort study

Core Tip: We aimed to evaluate the incidence of type 2 diabetes (T2DM) over time in women with polycystic ovary syndrome (PCOS) at different diagnosis ages, in comparison with non-PCOS controls. Our results showed that, among women diagnosed with PCOS at a young age, the incidence of T2DM was significantly higher than that of age-matched women in the general population. However, the risk disappeared among women diagnosed with PCOS after age 35. These findings highlight the importance of prompting a more aggressive treatment to prevent diabetes among women diagnosed with PCOS at a young age, and, in contrast, the lessened importance of this type of intervention in women diagnosed with PCOS at a late reproductive age.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine pathology that affects 5%-15% of women of reproductive age worldwide. The prevalence estimates vary according to the different diagnostic parameters applied[1]. It is also the most common cause of endocrine-related female infertility in the United States. This syndrome was first described by American gynecologists Irving F, Stein Sr. and Michael L Leventhal in 1935, when they reported a series of patients with enlarged polycystic ovaries, hirsutism, oligo/amenorrhea, and infertility. It has been demonstrated that PCOS includes a complex of systemic symptoms in addition to the reproductive apparatus. Over the last few decades, research studies have revealed that PCOS is strongly associated with metabolic disorders, including metabolic syndrome, obesity, insulin resistance, prediabetes, and type 2 diabetes (T2DM). The prevalence of metabolic syndrome in women with PCOS was approximately 6- to 7-fold higher than that detected in the controls[2,3]. According to a prospective case–control study, 64.4% of 271 patients with PCOS were noted to be insulin-resistant after adjusted for age, race, and body mass index (BMI)[4]. Based on clamp data, both obese and lean women with PCOS were more insulin-resistant compared with their weight-matched normal counterparts. In this study, insulin resistance (IR) was present in 75% of lean women with PCOS, 62% of overweight controls, and 95% of overweight women with PCOS[5]. Insulin resistance is defined as a reduced response of target tissues, such as the skeletal muscle and adipocytes. In women with PCOS, insulin-mediated glucose uptake is decreased by 35%-40% compared with age- and weight-comparable control women[6]. Because insulin resistance is the driving factor of hyperglycemia, women with POCS are particularly at risk of developing T2DM. The estimated prevalence of impaired glucose tolerance (IGT) and T2DM was 31%-37% and 7.5%-10.0%, respectively, in women with PCOS in the United States[7-9]. In two prospective trials of women with PCOS conducted in the United States and Turkey, after an average follow-up period of 2-3 years, both studies revealed a higher IGT and T2DM conversion rate compared with women without PCOS[9,10]. Abundant strong evidence supports the contention that diabetes is much more prevalent in women with PCOS than it is in the general population. We noticed that, even at a young age, women with PCOS also exhibit β-cell dysfunction, IGT, and T2DM[11,12]. Therefore, we aimed to evaluate the incidence of T2DM over time in women with PCOS at different diagnosis ages, in comparison with non-PCOS

controls. We selected the National Health Insurance Research Database (NHIRD), which records age, gender, diagnosis codes, comorbidities, and the clinical prescriptions for each beneficiary, as the data source.

MATERIALS AND METHODS

Data source

In this population-based retrospective cohort study, we used data from individuals in the Longitudinal Health Insurance Database 2000 (LHID2000), to evaluate the outcomes. LHID2000 is a subset of the NHIRD that contains the entire original claim data of 1000000 individuals randomly sampled from the 2000 registry for beneficiaries (ID) of the NHIRD, which maintains the registration data of everyone who was a beneficiary of the National Health Insurance (NHI) program during the period of 1996-2013. There are approximately 23.75 million individuals in this registry. The complete registration and claim data of these 1000000 individuals collected by the NHI program constitute the LHID2000. There was no significant difference in the gender distribution (χ^2 = 1.74, df = 1, *P* value = 0.187) between the patients in the LHID2000 and those in the original NHIRD[13]. The data recorded in the LHID2000 include demographic information; prescription details; clinical events; diagnosis codes in accordance with the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) system; and medical examinations and managements for all admitted patients and outpatients. In this study, we used LHID2000 from 1997 to 2010 as the research database, and followed to December 2013. This study was approved by the institutional review board of China Medical University in central Taiwan (CMUH104-REC2-115).

Study population and outcome assessment

In this retrospective cohort study, we aimed to compare women with PCOS with those without PCOS. We selected the PCOS cohort as follows: (1) Women older than 18 years of age and diagnosed with polycystic ovarian morphology; (2) Clinical visit for oligomenorrhea and/or anovulation problems, or hyperandrogenism problems at least twice a year; and (3) Women who underwent gynecological ultrasonography or blood testing for testosterone or 17-hydroxyprogesterone levels. Women were eligible to participate when all three conditions were met. Patients with the diagnoses of type 1 diabetes, T2DM, IGT, gestational diabetes, hyperinsulinism, Cushing's syndrome, and congenital adrenal hyperplasia before the date of initial PCOS diagnosis and those who were aged less than 18 years were excluded from the cohort. According to the inclusion and exclusion criteria, a total of 2545 people were defined as the PCOS group in this study (Figure 1). The factors associated with PCOS that are considered as potential confounding variables include lipid metabolism disorders, coronary artery disease, hypertension, chronic kidney disease, cerebrovascular accident, female infertility, obesity, chronic lymphocytic thyroiditis, major depression, and a history of anxiety before baseline. Prescriptions during follow-up for menstrual cycle regulation, ovulation induction, anti-androgen, and metformin were also considered potential confounding variables. The index date for the cohort group was assigned as the first time of recording of the ICD-9-CM code. The end point was set on the date of the new diagnosis of T2DM (more than three times at outpatient department or once in admission), the date of withdrawal from the NHI program, or the end of 2013. For the control group, women without PCOS were randomly selected and 1:1 frequency matched the cohort group by age, index date, and comorbidities. The comorbidities controlled in this study were lipid metabolism disorders, hypertension, coronary artery disease, chronic kidney disease, cerebrovascular accident, infertility, obesity, Hashimoto's disease, major depression, and anxiety.

Statistical analysis

The baseline characteristics of women with PCOS and controls are described by numbers and percentages. An intergroup comparison was performed using the chi-squared and *t*-test for categorical variables and continuous variables, respectively. The incidence rates of T2DM were calculated in person-years. We used univariable and multivariable Cox proportional hazard regression models to estimate and adjust the crude hazard ratio (HR). After adjustment for key covariates (age, comorbidity), we calculated the adjusted HR together with 95%CIs with statistical significance set at $P < 0.05$. Survival curves were estimated for each group, considered separately using the Kaplan–Meier method and compared statistically using the log-rank test. The Kaplan–Meier curves of the cumulative incidence of T2DM between the PCOS group and the control group were performed to estimate the cumulative probability of T2DM between two groups. All analyses were performed using the SAS software (version 9.4 for windows; SAS Institute, Cary, NC, United States).

The study was reviewed by our expert biostatistician Dr. Jing-Yang Huang.

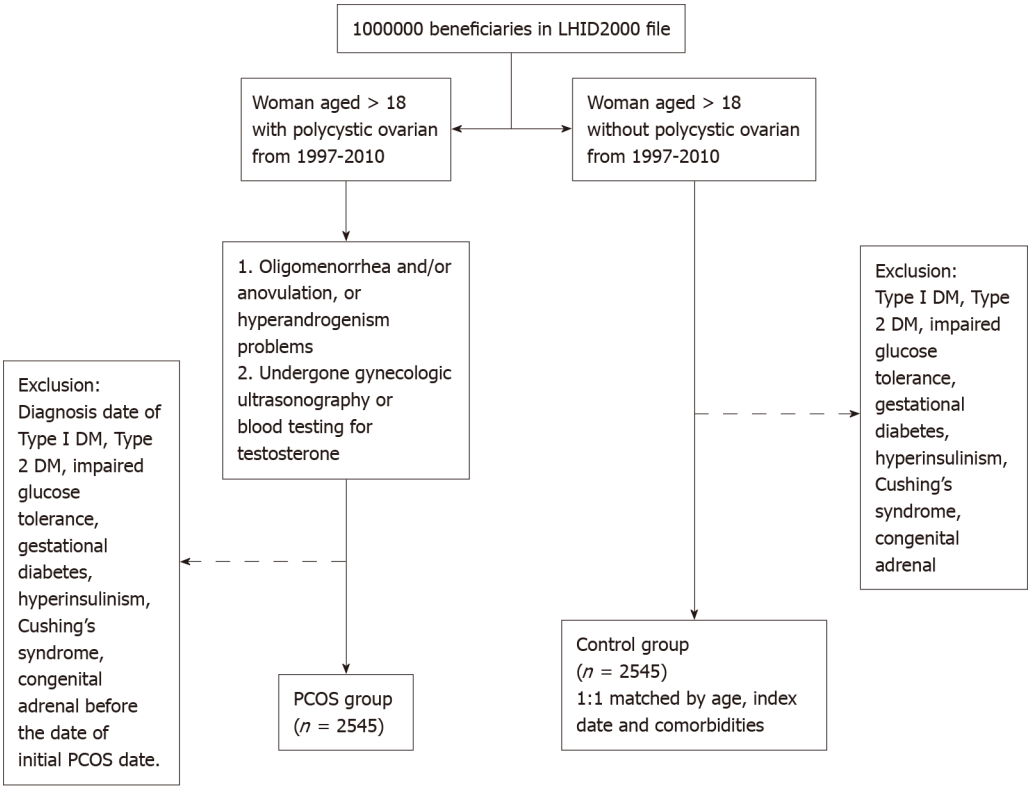

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Figure 1 Flow diagram of the recruitment of subjects from the 1 million samples randomly selected from the Taiwanese National Health Insurance Research Database from 1997 to 2010. LHID2000: Longitudinal Health Insurance Database 2000; PCOS: Polycystic ovary syndrome.

RESULTS

The first possible date for cohort entry (the study start date) was January 1, 1997, and patients could enter the cohort until December 31, 2010. The end of the follow-up time is December 2013. During the study period, we identified 2545 women with PCOS. These women were frequency matched at 1:1 to 2545 individuals in the non-POCS control group. The PCOS group and the control group were both followed for a mean period of 10 years, and the standard deviation was 3.14 *vs* 3.15 years (Table 1). In the baseline characteristic of the patients, all enrollees are stratified by age. The highest proportion of patients were into the 18-24-year age group (58.8%), followed by the 25-29-year age group (22.9%). The proportion of women over 30 years of age was only 18.3%. As expected, the age and comorbidity distributions of these two groups were similar because the groups were 1:1 propensity-score matched. Their mean age was 25 years, and there was no difference in the age stratification between the two groups. Women with PCOS were more likely to receive a prescription of metformin, oral contraceptive pills (OCPs), clomiphene citrate, and spironolactone.

In the PCOS group, the overall incidence of T2DM was 6.25 per 1000 person-years compared with 1.49 in the control group (Table 2). After adjustment for potential confounding variables (age, all comorbidities, and the medications listed in Table 1), the incidence of T2DM was higher in the PCOS group compared with the control group (HR = 5.13, 95%CI: 3.51-7.48, $P < 0.0001$). Moreover, the PCOS group showed a higher incidence of T2DM in the 18-24-year age group (HR = 10.4, 95%CI: 5.04-21.4, $P < 0.0001$). The incidence of T2DM decreased with the increasing diagnosis age. However, no such significant association was noted in women older than 35 years. All participants were stratified according to the presence or absence of comorbidities or of medication. Among women without comorbidities and no medication, the PCOS group exhibited a higher incidence of T2DM compared with the control group (non-comorbidity stratifications: adjusted HR = 7.62, 95%CI: 4.68-12.4; non-metformin stratifications: adjusted HR = 5.41, 95%CI: 3.67-7.98; non-OCP stratifications: adjusted HR = 5.18, 95%CI: 3.54-7.58; non-clomiphene stratifications: adjusted HR = 5.93, 95%CI: 3.94-8.92; non-spironolactone stratifications: adjusted HR = 5.07, 95%CI: 3.47-7.41). The Kaplan–Meier curves present the differences in the cumulative incidence of T2DM between the PCOS group and the control group (Figure 2). The cumulative incidence of T2DM in the PCOS group (dashed line) was significantly higher than that observed in the control group (solid line) (log-rank test, $P < 0.001$).

Table 1 Baseline patient characteristics

	PCOS				
	Yes (*n* = 2545)		No (*n* = 2545)		*P* value
	n	%	*n*	%	
Age, yr					> 0.99
18-24	1497	58.8	1497	58.8	
25-29	583	22.9	583	22.9	
30-34	289	11.4	289	11.4	
35-39	117	4.60	117	4.60	
40-44	45	1.77	45	1.77	
≥ 45	14	0.55	14	0.55	
mean ± SD	25.1 ± 5.81		25.2 ± 5.91		0.63
Comorbidity					
Disorders of lipid metabolism	38	1.49	38	1.49	> 0.99
Cardiovascular disease	3	0.12	8	0.31	0.13
Hypertension	27	1.06	27	1.06	> 0.99
Chronic kidney disease	2	0.08	4	0.16	0.41
Cerebrovascular accident	7	0.28	8	0.31	0.80
Infertility	151	5.93	151	5.93	> 0.99
Obesity	27	1.06	27	1.06	> 0.99
Hashimoto's disease	5	0.20	4	0.16	0.74
Major depression	18	0.71	27	1.06	0.18
Anxiety	169	6.64	169	6.64	> 0.99
Medication (during follow-up period)					
Metformin	238	9.35	18	0.71	< 0.0001
OCPs	443	17.4	72	2.83	< 0.0001
Clomiphene	1384	54.4	302	11.9	< 0.0001
Spironolactone	111	4.36	32	1.26	< 0.0001

Polycystic ovary syndrome group: follow-up time: 10.0; SD = 3.14. Control group: follow-up time: 10.0; SD = 3.16. PCOS: Polycystic ovary syndrome; OCPs: Oral contraceptive pills.

DISCUSSION

To our knowledge, this was the first attempt to analyze large-scale data to evaluate the relationship between women with PCOS and the development of T2DM in an East-Asian cohort. Moreover, this was the only study that stratified the cohorts into subgroups based on the age at diagnosis.

Our study found that, during a 10-year follow-up period, women with PCOS were associated with 5-fold higher risk of developing T2DM compared with women without PCOS. In past studies, the incidence of T2DM in women with PCOS presented with substantial clinical heterogeneity (ranging from 2- to 7-fold). There may be several explanations for these marked differences. First, different ethnic backgrounds may be responsible for the higher prevalence of T2DM. A small-size prospective trial carried out in the eastern Mediterranean region showed that 11.5% of women with PCOS and normal glucose tolerance (NGT) at the baseline converted to IGT with an annualized incidence rate of 4.5%. Furthermore, the annualized incidence rate from IGT converted to T2DM was 10.4%. In comparison, another similar study conducted in the United States reported that, among women with PCOS, the annualized conversion risk was 16% from NGT to IGT, and 2% from IGT to T2DM[9,10]. A nationwide population-based retrospective cohort study performed in Denmark found that the HR for women with PCOS who developed T2DM was 3.5 (95%CI: 3.2-3.8) when gestational diabetes mellitus was excluded. The results of the Danish study were slightly lower than our current findings (HR = 5.13, 95%CI: 3.51-

Table 2 Incidence rate and hazard ratio of type 2 diabetes between two groups stratified by gender, age, and comorbidity

	PCOS									
	No			Yes			Crude		Adjusted	
	Event	PY	IR	Event	PY	IR	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Overall	38	25483	1.49	159	25460	6.25	4.19 (2.94, 5.97)	< 0.0001	5.13 (3.51, 7.48)	< 0.0001
Age										
18-24	9	15453	0.58	74	15301	4.84	8.33 (4.17, 16.6)	< 0.0001	10.4 (5.04, 21.4)	< 0.0001
25-29	9	5630	1.60	40	5766	6.94	4.32 (2.10, 8.90)	< 0.0001	5.28 (2.42, 11.5)	< 0.0001
30-34	9	2753	3.27	27	2734	9.88	3.01 (1.42, 6.40)	0.004	4.06 (1.73, 9.53)	0.001
35-39	6	1117	5.37	11	1134	9.70	1.81 (0.67, 4.90)	0.24	2.14 (0.72, 6.35)	0.17
40-44	5	399	12.53	5	397	12.59	1.03 (0.30, 3.55)	0.97	1.68 (0.38, 7.41)	0.50
≥ 45	0	132	0.00	2	128	15.63				
Comorbidity[1]										
Yes	17	3864	4.40	32	3835	8.34	1.90 (1.06, 3.43)	0.03	2.14 (1.14, 3.99)	0.02
No	21	21620	0.97	127	21625	5.87	6.05 (3.81, 9.60)	< 0.0001	7.62 (4.68, 12.4)	< 0.0001
Medication										
Metformin										
Yes	2	191	10.47	21	2325	9.03	0.88 (0.21, 3.75)	0.86	0.54 (0.1, 2.78)	0.46
No	36	25292	1.42	138	23135	5.96	4.19 (2.91, 6.05)	< 0.0001	5.41 (3.67, 7.98)	< 0.0001
OCPs										
Yes	0	789	0.00	17	4559	3.73				
No	38	24695	1.54	142	20901	6.79	4.42 (3.09, 6.32)	< 0.0001	5.18 (3.54, 7.58)	< 0.0001
Clomiphene										
Yes	5	3349	1.49	72	14470	4.98	3.41 (1.38, 8.43)	0.008	3.26 (1.3, 8.21)	0.01
No	33	22135	1.49	87	10990	7.92	5.33 (3.57, 7.95)	< 0.0001	5.93 (3.94, 8.92)	< 0.0001
Spironolactone										
Yes	0	349	0.00	4	1211	3.30				
No	38	25135	1.51	155	24249	6.39	4.23 (2.97, 6.04)	< 0.0001	5.07 (3.47, 7.41)	< 0.0001

[1]Patients with any one of comorbidity were classified as the comorbidity group.

Models adjusted by age, all comorbidities and medications listed in Table 1. PY: Person-years; IR: Incidence rate, per 1000 person-years; HR: Hazard ratio; PCOS: Polycystic ovary syndrome; OCPs: Oral contraceptive pills.

7.48)[13]. The different ethnic backgrounds may be responsible for the higher prevalence of T2DM detected in Taiwan. A meta-analysis of multiple quality studies calculated an increased prevalence of IGT and T2DM among women with PCOS and different ethnicities (OR for IGT, Asia = 5.22, Americas = 4.4, Europe = 2.59)[14]. Genome-wide association studies (GWASs) have become a feasible option for studying the genetic background of PCOS, thus providing the ability of surveying a large number of genomes at once[15]. Two GWASs targeting PCOS have been performed in China; they identified 11 variants associated with PCOS risk in Han Chinese women who were diagnosed with PCOS (*i.e.*, who fulfilled all three Rotterdam criteria)[16,17]. However, not all loci for PCOS have been replicated in European women, which may speak to the variation in susceptible single-nucleotide polymorphisms (SNPs) among distinct racial and ethnic groups[18]. Some researchers believe that different combinations of SNPs may underlie the severity of the PCOS phenotypes, with Americans and Asians being more often characterized by the metabolic phenotype, and Europeans and Middle-Eastern women having a higher prevalence of hyperandrogenic phenotype[19]. Therefore, we assume that ethnicity may affect the transition from PCOS to diabetes[14].

Second, it may be related to the age at diagnosis of PCOS. This was also the most important finding of our study. There are indications that age may affect the incidence rate of conversion from PCOS to T2DM. According to a prospective study with a follow-up of 18 years performed in the United States, 53

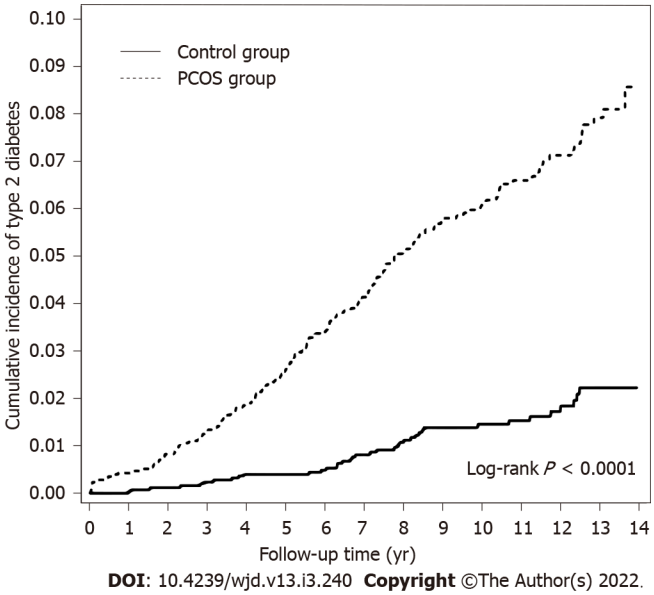

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Figure 2 Kaplan–Meier curves for the cumulative incidence of type 2 diabetes of the polycystic ovary syndrome and control groups.
PCOS: Polycystic ovary syndrome.

women fulfilled the criteria for PCOS at ages 20-32 (average, 26 years). Compared with those without PCOS, women with persistent PCOS had a 7-fold odds of developing diabetes[20]. Another 10-year follow-up study performed in China among women with PCOS aged 30-39 years reported that the age-standardized incidence rate of T2DM was approximately 3-fold compared with women without PCOS [21]. It is well established that the PCOS phenotype changes with aging, the improvement of phenotype, and oligo-ovulation, as indicated by the decrease in serum androgen levels (*e.g.*, testosterone, free androgen index, calculated free testosterone, androstenedione, and dehydroepiandrosterone sulfate) and increase in the number of regular menstrual cycles[22-25]. In healthy women, the positive correlation between age and worsening glucose tolerance is obvious after adjusting for BMI[26]. Interestingly, not only ovarian dysfunction and hyperandrogenism, but also insulin resistance, ameliorate during aging in women with PCOS[20]. According to a cross-sectional study, homeostasis model assessment (HOMA)-IR was negatively associated with age in women with PCOS as well as in different BMI subgroups, namely lean, normal-weight, and overweight subjects[27]. The observations that BMI and androgens are positively associated with HOMA-IR and that androgens decline with time suggest that these women achieved a better metabolic profile at their late reproductive ages. In a long-term prospective cohort study with a follow-up of more than 10 years, Kazemi Jaliseh *et al*[28] found that the adjusted HR for T2DM in women with PCOS aged ≤ 40 years was 4.9. In contrast, there was no difference between the two groups regarding the incidence rates of T2DM after the age of 40 years. The study included 178 women with PCOS and 1524 women without PCOS, and all PCOS cases were defined using the National Institutes of Health 1990 criteria, which carry the strongest clinical significance. The hazard differences between women with PCOS and those in the general population disappeared in their late reproductive years, which is in line with the results of the current study. Women who were diagnosed with PCOS before the age of 25 were 10 times more likely to develop T2DM compared with women without PCOS after adjusting for variance. The risk of developing T2DM subsequent to PCOS decreased with increasing diagnosis age: the adjusted HR was 10.4 in the 18-24-year age group, 5.28 in the 25-29-year age group, and 4.06 in the 29-34-year age group. Although the risk decreased with increasing age, it remained higher compared with that detected in women without PCOS. After age 35, the association between PCOS and T2DM was not statistically significant. Furthermore, among women without comorbidities and taking no medications, the incidence of T2DM was higher in the PCOS group than that in the control group. Several reasons for this result have been identified. First, women with PCOS who had no comorbidities showed a higher incidence of T2DM than the overall average, which means that the health problems caused by PCOS may be higher than previously recognized. Second, women without comorbidities and taking no medications may be relatively younger, which corroborates the previous assumption that women who are diagnosed with PCOS at a young age are more likely to develop T2DM. However, the sample size in the stratification of no medication is notably very small and may not provide reliable estimates and conclusive results.

The strength of our study consisted in the fact that NHIRD is one of the largest and most comprehensive nationwide population reimbursement databases in the world, as it covers almost 23 million residents in Taiwan with universal coverage. It provides a big sample size and complete records of medical visits and treatment, which are conducive to a longitudinal study design and age stratification.

Furthermore, research conducted using NHIRD can avoid a selection bias and the possibility of recall bias in questionnaire assessments.

The limitation of this study was that certain prognostic factors that are associated with the incidence of T2DM are not available through the NHIRD; namely, BMI, waist-hip ratio, lifestyle, and the results of blood tests (androgen and plasma glucose levels). Thus, we were unable to rule out the possibility that the differences in HR detected between the two groups stemmed from these factors. Moreover, NHI covers 96%-99% of Taiwan's population and 93% of hospitals and clinics are NHI-contracted. It subsidizes most medical treatments at a relatively low cost. However, there is still a possibility that patients reviewed in this study might have consulted other doctors before entering the NHI system. In addition, the sample size of the groups of women diagnosed with PCOS after the age of 35 years was relatively small, which may have led to imprecise estimates and statistical significance. Finally, the study population was homogeneous because all women were Asian. Therefore, additional research is required to substantiate this association among non-Asian women as well.

CONCLUSION

The data supplied here were from a relatively large population, spanning a long period. Our results showed that, among women diagnosed with PCOS at a young age, the incidence of T2DM was significantly higher than that of age-matched women in the general population. However, the risk disappeared among women diagnosed with PCOS after age 35. These findings highlight the importance of prompting a more aggressive treatment to prevent diabetes among women diagnosed with PCOS at a young age, and in contrast, the lessened importance of this type of intervention in women diagnosed with PCOS at a late reproductive age.

ARTICLE HIGHLIGHTS

Research background

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age. Research over the last few decades has revealed that PCOS is strongly associated with metabolic disorders. Even at a young age, women with PCOS also exhibit β-cell dysfunction, impaired glucose tolerance, and type 2 diabetes (T2DM).

Research motivation

Although current evidence supports the contention that diabetes is much more prevalent in women with PCOS than it is in the general population. The majority of longitudinal studies regarding the incidence of T2DM in women with PCOS are from non-Asian countries.

Research objectives

We aimed to evaluate the incidence of T2DM over time in women with PCOS at different diagnosis ages, in comparison with non-PCOS controls.

Research methods

The data retrieved from the Longitudinal Health Insurance Database 2000 (LHID2000). LHID2000 is a subset of the National Health Insurance Research Database (NHIRD) that contains the entire original claim data of 1000000 individuals randomly sampled from the 2000 registry for beneficiaries (ID) of the NHIRD, which maintains the registration data of everyone who was a beneficiary of the National Health Insurance program.

Research results

After adjustment for potential confounding variables (age, comorbidities and medications), the overall incidence of T2DM was higher in the PCOS group compared with the control group (HR = 5.13, 95%CI: 3.51-7.48, $P < 0.0001$). The risk of developing T2DM subsequent to PCOS decreased with increasing diagnosis age: the adjusted HR was 10.4 in the 18-24-year age group, 5.28 in the 25-29-year age group, and 4.06 in the 29-34-year age group. After age 35, the association between PCOS and T2DM was not statistically significant.

Research conclusions

The risk of developing T2DM subsequent to PCOS decreased with increasing diagnosis age. No such significant association was noted in women older than 35 years.

Research perspectives

These findings highlight the importance of prompting a more aggressive treatment to prevent diabetes among women diagnosed with PCOS at a young age, and, in contrast, the lessened importance of this type of intervention in women diagnosed with PCOS at a late reproductive age.

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FOOTNOTES

Author contributions: Liao WT and Wu CC contributed to the conceptualization, writing-review and editing; Huang JY, Lee MT and Yang YC contributed to the methodology; Yang YC contributed to the software and formal analysis; Liao WT and Lee MT contributed to the validation; Huang JY contributed to the data curation; Liao WT contributed to the writing-original draft preparation; Wu CC contributed to the supervision and project administration; all authors reviewed the final version of the manuscript.

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Informed consent statement: The informed consent form is waived by the Institutional Review Board.

Conflict-of-interest statement: The authors declare no conflict-of-interest.

Data sharing statement: Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality and because they were used under license. Upon request, the corresponding author will provide details on the restrictions and conditions under which access to some data may be provided.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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ORIGINAL ARTICLE

Retrospective Study

Hemoglobin within normal range is negatively related to hemoglobin A1c in a nondiabetic American population aged 16 years and older

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Abstract

BACKGROUND

Protein glycosylated hemoglobin, hemoglobin A1c (HbA1c) binds hemoglobin (Hb) in red blood cells to blood glucose. However, the relationship between Hb and HbA1c remains unclear.

AIM

To elucidate their relationship in a nondiabetic population aged ≥ 16 years in the United States, using data from the 1999-2018 National Health and Nutrition Examination Survey.

METHODS

This study was based on data from 44560 adults aged ≥ 16 years, excluding those with diabetes. The relationship was estimated using a multivariate regression. We also used piecewise linear regression for subgroup analysis based on age and sex stratification and analysis of the threshold effects of Hb on HbA1c.

RESULTS

Hb and HbA1c levels were negatively correlated in the unadjusted model (β = -0.01; 95%CI: -0.01, -0.01). The correlation was significantly negative when the regression model was minimally regulated and stratified by age and sex, and remained negative when the model was further regulated (more than 10%) to identify covariates with the HbA1c level influence estimates. In subgroup analyses based on age and sex stratification, the association remained negative when the covariates were controlled. A nonlinear relationship was observed between them when the Hb levels reached the tipping point (13.2 g/dL) (adjusted odds ratio, -0.04; 95%CI: -0.05, -0.03) and when the Hb levels exceeded 13.2 g/dL (adjusted odds ratio, -0.10; 95%CI: -0.10, -0.09).

CONCLUSION
Our study shows that normal Hb levels are negatively correlated with HbA1c in nondiabetic Americans aged ≥ 16 years.

Key Words: Haemoglobin; Glycosylated haemoglobin; Diabetes; National Health and Nutrition Examination Survey

Core Tip: Our research revealed that hemoglobin (Hb) within the normal values is negatively related to hemoglobin A1c (HbA1c) in non-diabetic American populations aged 16 years and older. HbA1c decreases by 0.08% for every 1g/dL increase in Hb.

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INTRODUCTION

Diabetes mellitus (DM) has a high global incidence. The prevalence and incidence of DM continue to increase annually. DM is a major cause of global morbidity and mortality, and was one of the major causes of death in the United States in 2015. Over 30 million and 86 million Americans suffer from diabetes and prediabetes, respectively, which could increase the occurrence rate of many chronic diseases, especially type 2 DM (T2DM)[1]. Obesity may serve as a major inducement factor for diabetes, and the prevalence of diabetes and obesity are increasing[2]. Diabetes status can be classified into three categories: nondiabetes, prediabetes, and diabetes (T2DM)[3]. Chronic prediabetes and diabetes often cause a series of complications, including renal, ophthalmological, neurological, and vascular complications. It is well known that controlling high blood glucose levels could reduce and postpone the appearance and progression of DM-related complications[4]. Therefore, many prospective ongoing clinical studies are evaluating the efficacy of new and rarely studied diabetes biomarkers.

Hemoglobin (Hb) is a protein molecule that only exists in red blood cells (RBCs) that can bind oxygen. In the bloodstream, Hb is glycated. Hemoglobin A1c (HbA1c) acts as glycosylated hemoglobin (GHb) constructed by the nonenzymatic binding of glucose to valine at the N-terminus of the Hb β chain, which is the most abundant and common Hb in human erythrocytes. The GHb (HbA1c) level represents the percentage of Hb proteins bound to glucose. Glycemic control has been assessed using GHb. The higher the primary environmental level of blood glucose, the higher the HbA1c level[5]. However, the relationship between the Hb and HbA1c levels remains unclear. Hence, our study aimed to reveal the relationship between the normal level of Hb and GHb in a nondiabetic American population aged ≥ 16 years through cross-sectional investigation data obtained from the 1999-2018 National Health and Nutrition Examination Survey (NHANES).

MATERIALS AND METHODS

Population research

This study analyzed the NHANES data from 1999 to 2018 (20 years). The NHANES participants are representative of the non-institutionalized civilians in America employed by the NHANES multistage stratified sampling design[6].

A total of 101317 participants were registered in the NHANES 1999-2018 database. In this research, 44560 adults aged ≥ 16 years with Hb and HbA1c level data were considered available. We excluded the individual cases with missing HbA1c data (n = 36364); with missing Hb data (n = 110); with missing body mass index (BMI) data (n = 1031); aged < 16 years (n = 7,082); diagnosed with diabetes (n = 5923); with an HbA1c level of > 6.5% (n = 1461) or a glucose level of > 7.0 mol/L (n = 1906); and with an abnormal Hb level (n = 2880). Furthermore, 44560 nondiabetic patients were included in the final analysis (Figure 1).

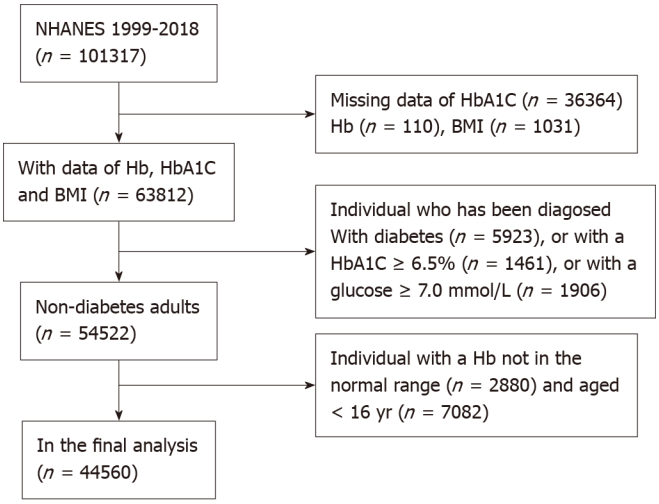

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Figure 1 Flowchart of study participants. NHANES: National Health and Nutrition Examination Survey; HbA1C: Hemoglobin A1c; BMI: Body mass index.

Variable research

The exposure variable was the Hb level. The method used to derive the complete blood count (CBC) parameters was based on the Beckmann Kurt counting and grading method, combined with an automatic dilution and hybrid device used for sample treatment and a single beam photometer for the determination of the Hb level.

The outcome variable was the HbA1c level. The HbA1c whole blood sample was processed, stockpiled, and transferred to the University of Kansas, Columbia, Missouri.

The multivariate model contained variables that may confuse the association between the HbA1c and Hb. Age; sex; ethnicity; education level; marital status; smoking behavior; BMI; levels of blood glucose, uric acid, total protein, alanine aminotransferase, cholesterol, and serum creatinine; platelet count; and white blood cell and RBC counts were acquired from questionnaires. Smoking behavior was derived from the question "Have you/Has SP smoked more than 100 cigarettes in your/his/her whole life?" The variable name was SMQ020. The SAS label referred to having smoked more than 100 cigarettes in a lifetime. Marital status was defined as follows: unmarried (never married), married (including married and living with partner), divorced (including widowed, divorced, and separated), and unknown (including refused, unknown, and missing). Educational level was quantified as follows: less than high school (including less than the 9th grade and 9th to 11th grade), high school, and above high school (including some university or AA degrees and above university). Race was classified as follows: Mexican Americans, non-Hispanic whites, non-Hispanic blacks, Hispanics, and others, including multiple ethnic groups. BMI was measured at the mobile examination center and calculated as weight/height^2. Blood glucose, total protein, uric acid, cholesterol, alanine aminotransferase, and serum creatinine levels and other data were obtained through the Beckman Synchron LX20 standard biochemical curve analysis. The platelet and white blood cell/RBC count data were obtained from a CBC with a 5-part differential. Diabetes was defined as an HbA1c level of > 6.5% or a fasting blood glucose level of > 7 mmol/L, according to the 2019 American Diabetes Association standards[7]. Details of the data are presented in Table 1 and freely available on the NHANES website (www.cdc.gov/nchs/nhanes/).

Further subgroup analyses were performed based on sex and age. The quartile classification of the Hb level and sensitivity analysis was performed, and the trend P value was calculated. This was followed by a subgroup analysis sorted by age and sex. We applied smoothing curve fitting and generalized additive models to search for the potential nonlinear relationship between the GHb and Hb levels. Piecewise linear regression was employed to analyze the threshold effect of HbA1c. All analyses incorporated the NHANES sampling weights. Statistical significance was set at $P < 0.05$.

RESULTS

A total of 44560 participants ≥ 16 years of age were included in this study. The weighted distributions of sex are shown in Table 1. Women were more educated than men and comprised a higher percentage of non-Hispanic whites and older age individuals; they also had higher cholesterol levels and white blood cell and platelet counts, lower percentage of smoking at least 100 cigarettes in a lifetime, lower levels of alanine aminotransferase, total protein, serum uric acid, serum creatinine, blood glucose, Hb, and HbA1c, and a lower RBC count.

Table 1 Characteristics of participants in the present work

	Male (*n* = 22255)	Female (*n* = 22305)	*P* value
Age (yr)	42.12 ± 16.96	43.92 ± 17.71	< 0.0001
Race/ethnicity (%)			< 0.0001
Non-Hispanic White	68.87	70.50	
Non-Hispanic Black	9.90	9.51	
Mexican American	9.07	7.61	
Other race/ethnicity	12.16	12.38	
Education level (%)			< 0.0001
Less than high school	15.25	13.56	
High school	22.89	21.07	
More than high school	53.71	58.36	
Others	8.15	7.01	
Marital status			< 0.0001
Never married	22.22	17.57	
Married	61.53	57.48	
Divorced	10.22	19.85	
Others	6.03	5.10	
Smoked at least 100 cigarettes in life (%)			< 0.0001
Yes	48.27	37.03	
No	44.76	56.91	
Others	6.97	6.06	
Body mass index (kg/m^2)	27.91 ± 5.70	27.95 ± 7.01	0.4535
Alanine aminotransferase (U/L)	29.29 ± 22.06	20.74 ± 20.30	< 0.0001
Serum creatinine (μmol/L)	85.93 ± 26.19	66.31 ± 18.96	< 0.0001
Blood glucose (mmol/L)	5.08 ± 0.61	4.95 ± 0.59	< 0.0001
Total protein (g/L)	72.34 ± 4.53	71.24 ± 4.62	< 0.0001
Uric acid (μmol/L)	361.26 ± 72.02	277.45 ± 68.64	< 0.0001
Cholesterol (mmol/L)	5.0 ± 1.1	5.1 ± 1.1	< 0.0001
White blood cell count (10^9/L)	7.10 ± 2.30	7.30 ± 2.21	< 0.0001
Red blood cell count (10^{12}/L)	4.99 ± 0.41	4.48 ± 0.36	< 0.0001
Platelet count (10^9/L)	240.75 ± 57.14	266.28 ± 64.99	< 0.0001
Hemoglobin (g/dL)	15.28 ± 1.02	13.64 ± 0.93	< 0.0001
Hemoglobin A1c (%)	5.34 ± 0.36	5.32 ± 0.37	< 0.0001

Data are show in mean ± SD, including age, body mass index, alanine aminotransferase, cholesterol, creatinine, blood glucose, red/white blood cell count, platelet count, total protein, serum uric acid, hemoglobin, and hemoglobin A1c. Weighted linear regression model was employed to compute the *P* value. Categorical variables are represented in percentage (%), including race, educational level, marital status, smoking (> 100 cigarettes in life), while weighted chi-square test was employed to compute the *P* value.

The correlation between Hb and HbA1c obtained by multiple regression analysis is shown in Table 2. There was a negative correlation between the Hb and HbA1c levels (β = -0.01; 95%CI: -0.01, -0.01) in the unadjusted model. The correlation remained significant with the smallest adjustment for age and sex in the regression model (β = -0.01; 95%CI: -0.01, -0.00). After further adjusting the covariates with the estimated impact of the HbA1c level in the model exceeding 10%, the correlation remained negative (β = -0.08; 95%CI: -0.08, -0.07). *P* value was < 0.001 for trend.

Table 2 Relation between hemoglobin (1 g/dL) and hemoglobin A1c level (%)

	Unadjusted model β (95%CI)	Minimally adjusted model β (95%CI)	Fully adjusted model β (95%CI)
Hemoglobin	-0.01 (-0.01, -0.01)[c]	-0.01 (-0.01, -0.00)[c]	-0.08 (-0.08, -0.07)[c]
Hemoglobin (Quartile)			
Q1	Reference	Reference	Reference
Q2	0.01 (0.00, 0.02)	0.01 (-0.00, 0.01)	-0.06 (-0.07, -0.05)[c]
Q3	0.02 (0.01, 0.03)[b]	0.01 (-0.00, 0.02)	-0.12 (-0.13, -0.11)[c]
Q4	-0.04 (-0.05, -0.03)[c]	-0.02 (-0.03, -0.01)[c]	-0.23 (-0.24, -0.22)[c]
P for trend	< 0.001	< 0.001	< 0.001

[a]*P*< 0.01.
[b]*P*< 0.001.
[c]*P*< 0.0001.
Three models were employed to analyze the relation in this work, namely, unadjusted model, minimally adjusted model, and fully adjusted model. No covariates were regulated in unadjusted model. Only age and gender were regulated in minimally adjusted model. Lastly, in fully adjusted model, all parameters were adjusted, including age, gender, ethnicity, educational level, marital status, smoking behavior, body mass index, uric acid, total protein, serum creatinine, blood glucose, alanine aminotransferase, cholesterol, platelet count, and red/ white blood cell count.

The correlation was still negative in the subgroup analysis classified by age (16-29 years, β = -0.011; 95%CI: -0.015, -0.008; 30-51 years, β = -0.004; 95%CI: 0.008, 0.001; 52-85 years, β = -0.021; 95%CI: -0.025, -0.016) and sex (men, β = -0.057; 95%CI: -0.062, -0.052; women, β = -0.012; 95%CI: -0.017, -0.007) when the covariates were controlled. The results are presented in Table 3. The smooth curve fitting and generalized additive model further verified the negative correlation between the Hb and HbA1c levels (Figures 2-4).

A nonlinear relationship between Hb and HbA1c was observed when the Hb levels reached the turning point (13.2 g/dL) [adjusted odds ratio (OR), -0.04; 95%CI: -0.05, -0.03; $P < 0.0001$) and when the Hb levels exceeded 13.2 g/dL (adjusted OR, -0.10; 95%CI: -0.10, -0.09; $P < 0.0001$). The results are presented in Table 4.

DISCUSSION

In the present study, we examined numerous samples of American nondiabetic individuals aged ≥ 16 years to investigate the relationship between the Hb and HbA1c levels in the normal range. Our study showed that the Hb and HbA1c levels were negatively correlated in both men and women.

HbA1c is a GHb, which is a nonenzymatic reaction of glucose binding to Hb. HbA1c is considered as a better marker to evaluate the state of DM compared with blood glucose monitoring, and it is stable and able to represent the average blood glucose level over the past 2-3 mo. The HbA1c levels are affected by a large number of factors, such as race, RBC disorders, and hemoglobinopathies.

A large retrospective cohort study conducted by Grossman *et al* included 11,352 individuals without diabetes and assessed the correlation between the Hb and HbA1c levels. The fifth highest Hb level of HbA1c individuals was significantly lower than that of the other fifth of individuals, and the correlation between the Hb and HbA1c levels was negligible[8]. However, Lai *et al*[9] found that in 1659 Chinese nondiabetic adults aged 20-49 years, the normal Hb levels were negatively correlated with HbA1c.

In our study, we found that the correlation between the Hb and HbA1c levels was obviously negative in the unadjusted model. When minimal adjustments to sex and age were made in the regression model and when the model further adjusted for the estimated value of the HbA1c level to exceed 10 covariates, the association was still significant. Our results were generally consistent with those of Lai *et al*[9]'s studies on Chinese populations.

Since 1999, the NHANES has investigated approximately 5000 people in 15 different counties in America every year[8,10-13]. Each participant represents approximately 65000 people across the country, and such individuals have made significant contributions to the study. Our research had a large-scale, population-based research design; therefore, our results can be extended to the entire American population. However, there are some limitations that should be noted. First, a cause-and-effect relationship between Hb and HbA1c levels could not be established as a consequence of the cross-sectional design of our research. Longitudinal, prospective, large-scale human studies are needed at this point. Second, some covariant data were extracted from self-reporting, which may be easily affected by self-reporting bias. Nevertheless, the data were gathered by skilled interviewers in accordance with

Table 3 Age- and sex-stratified analysis of correlation for hemoglobin (g/dL) and hemoglobin A1c (%)

	Unadjusted model β (95%CI)	Minimally adjusted model β (95%CI)	Fully adjusted model β (95%CI)
Stratified by age			
16-29	-0.011 (-0.015, -0.008)[a]	-0.054 (-0.059, -0.048)[a]	-0.104 (-0.110, -0.097)[a]
30-51	-0.004 (-0.008, 0.001)	-0.037 (-0.042, -0.031)[a]	-0.090 (-0.097, -0.083)[a]
52-85	-0.021 (-0.025, -0.016)[a]	-0.023 (-0.028, -0.017)[a]	-0.084 (-0.091, -0.077)[a]
Stratified by sex			
Men	-0.057 (-0.062, -0.052)[a]	-0.031 (-0.036, -0.027)[a]	-0.085 (-0.091, -0.080)[a]
Women	-0.012 (-0.017, -0.007)[a]	-0.033 (-0.038, -0.029)[a]	-0.091 (-0.096, -0.085)[a]

[a]$P < 0.0001$.

Three models were employed to analyze the relation in this work, namely, unadjusted model, minimally adjusted model, and fully adjusted model. No covariates were regulated in unadjusted model. Only age was regulated in minimally adjusted model. Lastly, in fully adjusted model, all parameters were regulated, including age, gender, ethnicity, educational level, marital status, smoking behavior, body mass index, uric acid, total protein, serum creatinine, blood glucose, alanine aminotransferase, cholesterol, platelet count, and red/ white blood cell count.

Table 4 Threshold effect analysis of hemoglobin-on-hemoglobin A1c using piecewise linear regression

Point of hemoglobin (g/dL)	Odd ratio (95%CI)	*P* value
< 13.2	-0.04 (-0.05, -0.03)	< 0.0001
> 13.2	-0.10 (-0.10, -0.09)	< 0.0001

A threshold of 13.2g/dL for the hemoglobin existed for hemoglobin A1c. Parameters were adjusted, including age, race, body mass index, smoking (> 100 cigarettes in life), educational level, marital status, serum uric acid, alanine aminotransferase, creatinine, blood glucose, total protein, cholesterol, red/white blood cell count, and platelet count.

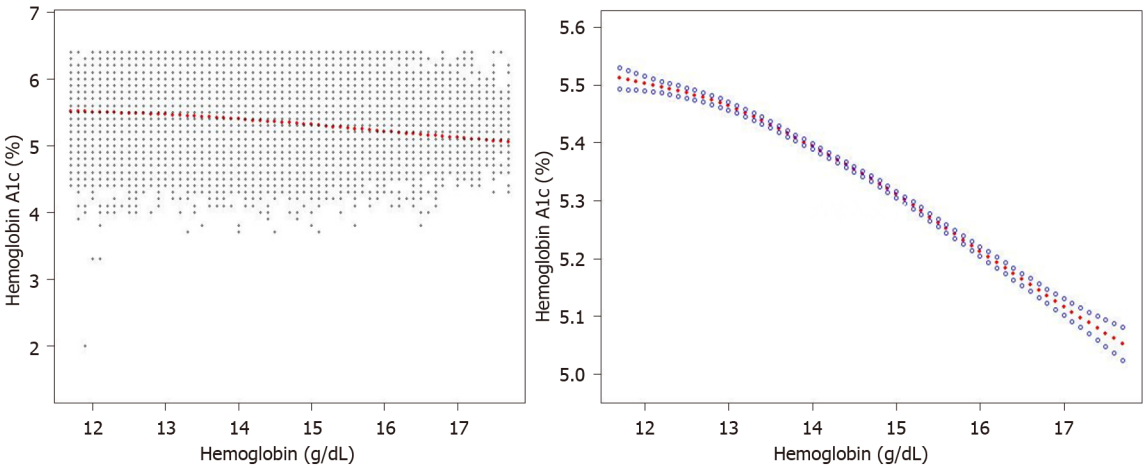

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Figure 2 Relation between hemoglobin and hemoglobin A1c among participants. Samples are shown in black points and smooth curve fitting data points is represented in solid red line. Besides, the 95%CI is represented in blue band. All parameters were modified, including age, gender, ethnicity, educational level, marital status, blood glucose, smoke behavior, body mass index, uric acid, total protein, alanine aminotransferase, cholesterol, serum creatinine, platelet count, and red/white blood cell count.

standardized agreements. Third, we excluded individuals with diabetes and abnormal Hb levels and those younger than 16 years of age. Therefore, our conclusions do not apply to these groups of people. Fourth, although several potential confounding factors were regulated, other potential confounding factors were not included in this study. Therefore, our study may include biases. Further prospective studies with large sample sizes are needed which include the measurement of these additional variables.

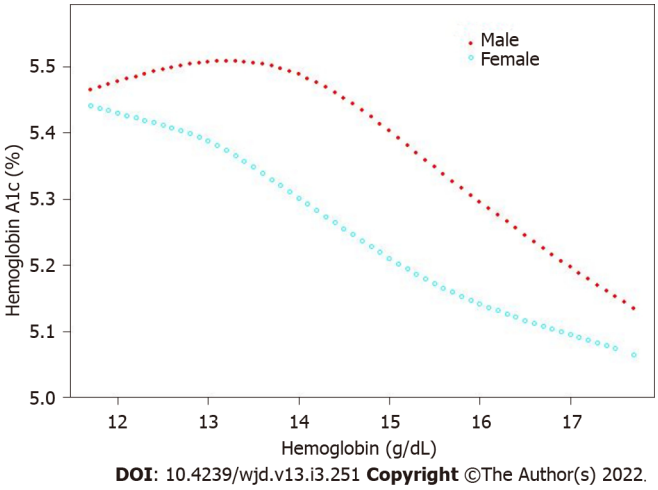

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Figure 3 Sex-stratified analysis of correlation for hemoglobin and hemoglobin A1c. All parameters were regulated, including age, ethnicity, educational level, marital status, blood glucose, smoking behavior, body mass index, uric acid, total protein, alanine aminotransferase, cholesterol, serum creatinine, platelet count and red/white blood cell count.

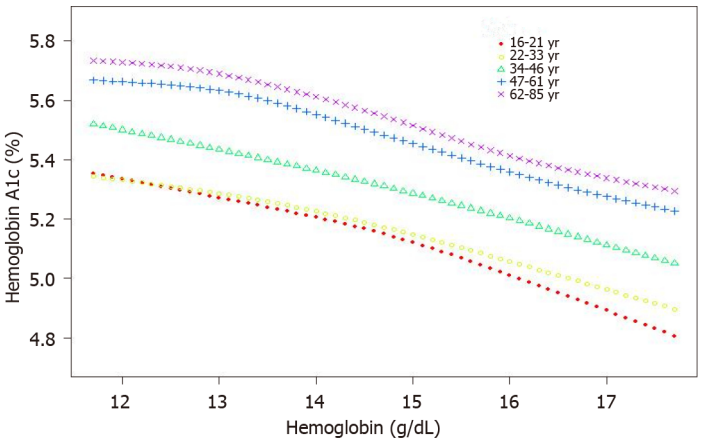

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Figure 4 Age-stratified analysis of correlation for hemoglobin and hemoglobin A1c in five groups. All parameters were adjusted, including sex, race, educational level, marital status, blood glucose, smoking behavior, body mass index, uric acid, total protein, alanine aminotransferase, cholesterol, serum creatinine, platelet count, and red/white blood cell count.

CONCLUSION

In conclusion, our results show that in the nondiabetic American population aged ≥ 16 years, the Hb levels were negatively correlated with the HbA1c levels within the normal range in both men and women. The Hb levels were independent and negatively related to the HbA1c levels.

ARTICLE HIGHLIGHTS

Research background

The relationship between hemoglobin (Hb) and hemoglobin A1c (HbA1c) remains unclear.

Research motivation

To elucidate the relationship between Hb and HbA1c in a nondiabetic population aged ≥ 16 years in America.

Research objectives

To elucidate the relationship between Hb and HbA1c.

Research methods
The relationship was estimated using a multivariate regression.

Research results
Hb levels are negatively correlated with HbA1c.

Research conclusions
Normal Hb levels are negatively correlated with HbA1c in nondiabetic Americans aged ≥ 16 years.

Research perspectives
From a clinical point of view, HbA1c decreases by 0.08% for every 1 g/dL increase in Hb.

FOOTNOTES

Author contributions: Bai XF sorted out the data and wrote the draft; Wang H and Zhao QL revised the article.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: The data that support the findings of this study are openly available by contacting the corresponding author.

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ORIGINAL ARTICLE

Observational Study

Age at diagnosis of type 2 diabetes and cardiovascular risk factor profile: A pooled analysis

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Abstract

BACKGROUND

The diagnosis of type 2 diabetes (T2D) in younger adults, an increasingly common public health issue, is associated with a higher risk of cardiovascular complications and mortality, which may be due to a more adverse cardiovascular risk profile in individuals diagnosed at a younger age.

AIM

To investigate the association between age at diagnosis and the cardiovascular risk profile in adults with T2D.

METHODS

A pooled dataset was used, comprised of data from five previous studies of adults with T2D, including 1409 participants of whom 196 were diagnosed with T2D under the age of 40 years. Anthropometric and blood biomarker measurements included body weight, body mass index (BMI), waist circumference, body fat percentage, glycaemic control (HbA1c), lipid profile and blood pressure. Univariable and multivariable linear regression models, adjusted for diabetes duration, sex, ethnicity and smoking status, were used to investigate the association between age at diagnosis and each cardiovascular risk factor.

RESULTS

A higher proportion of participants diagnosed with T2D under the age of 40 were female, current smokers and treated with glucose-lowering medications, compared to participants diagnosed later in life. Participants diagnosed with T2D under the age of 40 also had higher body weight, BMI, waist circumference and body fat percentage, in addition to a more adverse lipid profile, compared to participants diagnosed at an older age. Modelling results showed that each one year reduction in age at diagnosis was significantly associated with 0.67 kg higher body weight [95% confidence interval (CI): 0.52-0.82 kg], 0.18 kg/m^2 higher BMI (95%CI: 0.10-0.25) and 0.32 cm higher waist circumference (95%CI: 0.14-0.49), after adjustment for duration of diabetes and other confounders. Younger age at diagnosis was also significantly associated with higher HbA1c, total cholesterol, low-density lipoprotein cholesterol and triglycerides.

CONCLUSION

The diagnosis of T2D earlier in life is associated with a worse cardiovascular risk factor profile, compared to those diagnosed later in life.

Key Words: Type 2 diabetes mellitus; Early-onset adult type 2 diabetes; Age of onset; Cardiovascular risk; Young adults; Glycaemic control; Obesity

Core Tip: The diagnosis of type 2 diabetes (T2D) in younger adults, an increasingly common public health issue, is associated with a higher risk of cardiovascular complications and mortality, which may be due to a more adverse cardiovascular risk profile in individuals diagnosed at a younger age. This analysis demonstrates the adverse effect of younger diagnosis of T2D on cardiovascular risk factors, highlighting the need for targeted multifactorial age-appropriate interventions in order to improve the cardiovascular risk factor profile of younger adults with T2D and reduce their subsequent risk of cardiovascular complications and mortality.

Citation: Barker MM, Zaccardi F, Brady EM, Gulsin GS, Hall AP, Henson J, Htike ZZ, Khunti K, McCann GP, Redman EL, Webb DR, Wilmot EG, Yates T, Yeo J, Davies MJ, Sargeant JA. Age at diagnosis of type 2 diabetes and cardiovascular risk factor profile: A pooled analysis. *World J Diabetes* 2022; 13(3): 260-271
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INTRODUCTION

Type 2 diabetes (T2D), a significant and increasing public health issue, was tradi-tionally considered a disease of mid- to late adulthood[1]. However, the prevalence of T2D among younger adults (*e.g.*, diagnosed < 40 years of age; "early-onset adult T2D") has rapidly increased over the last few decades, now constituting between 15%-20% of all adults with T2D worldwide[2-4].The diagnosis of T2D at an earlier age is associated with an increased relative risk of mortality and of both microvascular and macrovascular complications, as highlighted by a recent meta-analysis of 26 studies[5]. Previous studies have suggested that a more adverse cardiovascular risk profile in adults diagnosed with T2D at a younger age [including higher glycaemic control (HbA1c), higher prevalence of obesity and a worse lipid profile] may explain some of the increased risk of mortality and complications observed among younger adults with T2D[3,6-9]. However, some conflicting results emerged within these studies[3,6,8,

9], whilst most have investigated the effect of age at diagnosis as a categorical variable (early- *vs* later-onset T2D). Consequently, estimates for the difference in risk factors incurred by each one year reduction in age at diagnosis are sparse[3].

Given the increase in prevalence of early-onset T2D and the higher risk of mortality and cardiovascular complications observed in younger adults with T2D, a comprehensive understanding of the effect of diagnostic age on cardiovascular risk factor profile is crucial. This analysis aimed to investigate the association between age at diagnosis, as a continuous variable, and the cardiovascular risk profile of adults with T2D, including measures of adiposity, HbA1c, lipid metabolism and blood pressure, using a pooled dataset of research trial data from multi-ethnic study populations in the United Kingdom.

MATERIALS AND METHODS

Pooled dataset

This analysis used a pooled dataset, comprising data from five previous or ongoing studies of adults with T2D in the United Kingdom: Chronotype of Patients with Type 2 Diabetes and Effect on Glycaemic Control (CODEC)[10], Effects of Liraglutide in Young Adults With Type 2 Diabetes (LYDIA), Early Detection of Cardiac Dysfunction and Health Behaviours in the Young with Type 2 Diabetes (EXPEDITION)[11], Diabetes Interventional Assessment of Slimming or Training to Lessen Inconspicuous Cardiovascular Dysfunction (DIASTOLIC)[12] and Prevalence and Determinants of Subclinical Cardiovascular Dysfunction in Adults with Type 2 Diabetes Mellitus (PREDICT)[13]. The rationale, design and eligibility criteria of these studies have been published previously, in addition to the main outcomes of the completed trials (LYDIA, EXPEDITION, DIASTOLIC)[10-14]. The aims, eligible age ranges and progress of each study are described in Table 1. Each study received ethical approval and all participants provided written informed consent. The pooled dataset used in the current analysis included all participants diagnosed at 16 years or older.

Outcome measurement

Outcome data used in this analysis were collected during baseline assessments within the pooled studies. During these baseline visits, information on demographics (including current age at visit), medical history (including age at T2D diagnosis) and medication use were collected. Anthropometric [including body weight, body mass index (BMI), waist circumference, and body fat percentage] and blood pressure measurements were collected using standardised procedures, and a blood sample was taken for measurement of routine circulating biomarkers (performed by accredited NHS clinical pathology laboratories using quality controlled enzymatic assays).

Statistical analysis

In order to compare demographic variables, cardiovascular complications, medication use, and cardiovascular risk factors [body weight, BMI, waist circumference, body fat percentage, HbA1c, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, systolic and diastolic blood pressure] by age at diagnosis, all data were first presented as median [interquartile range (IQR)] or percentages, as appropriate, using three diagnostic age categories: Diagnosed under 40 years of age, diagnosed between 40-59 years and diagnosed aged 60 years or older. Linear regression models were then used to investigate the association of age at diagnosis, used as a continuous variable, and each cardiova-scular risk factor. In order to assess the possibility of deviations from linearity, models were also conducted using a spline transformation of age at diagnosis for each cardiovascular risk factor. These models were compared to the linear models using Bayesian Information Criterion scores. For all models, there was no evidence of a significant difference between the spline and the linear models, therefore linear regression was used for the analyses.

As younger diagnosis may often predispose individuals to longer duration of T2D, it was important to assess whether any association between age at diagnosis and cardiovascular risk factors remained once diabetes duration was controlled for, as well as after adjustment for other important confounding variables. Therefore, three models were constructed for each cardiovascular risk factor: Model 1 (unadjusted univariable model), Model 2 (adjusted for duration of T2D alone), Model 3 (adjusted for duration of T2D, sex, ethnicity and smoking status). Robust standard errors were used to account for the clustering of data from the different studies.

RESULTS

Participant characteristics

In total, 1409 participants were included in the pooled dataset, of whom 196 (13.9%) were diagnosed with T2D under the age of 40 years, 846 (60.0%) were diagnosed between 40-59 years, and 367 (26.1%)

Table 1 Summary of studies included in the pooled dataset

Study name	Aim	Eligible age range (yr)	Exclusion criteria[1]	Clinical Trials.gov Registration Number	Ongoing/completed
CODEC	Observational study to investigate the effect of chronotype on glycaemic controls in adults with T2DM	18-75	N/A	NCT02973412	Ongoing
LYDIA	Randomised active-comparator trial to investigate the effect of liraglutide compared to sitagliptin on cardiac structure and function in younger adults with T2DM	18-60	Treatment with insulin, SGLT-2 inhibitors, GLP-1 receptor agonists of DPP-4 inhibitors; Active cardiovascular disease, including history of myocardial infarction within the past 6 mo and/or heart failure	NCT02043054	Completed
EXPEDITION	Observational study to phenotype younger adults with T2DM	18-40	N/A	N/A	Completed
DIASTOLIC	Randomised controlled trial to compare diet and exercise interventions to standard care in adults with T2DM	18-65	Current treatment with more than three glucose-lowering medications or insulin; Stroke, peripheral vascular disease, atrial fibrillation, heart failure/disease, angina	NCT02590822	Completed
PREDICT	Observational study to investigate the prevalence and determinants of subclinical cardiovascular dysfunction in adults with T2DM	18-75	Stroke, symptomatic peripheral vascular disease, atrial fibrillation, history of heart failure, history of myocardial infarction, moderate or severe heart valve disease, angina	NCT03132129	Ongoing

[1]Criteria related to cardiovascular complications or medications relevant to this analysis.

T2DM: Type 2 diabetes mellitus; SGLT-2: Sodium-glucose cotransporter-2; GLP-1: Glucagon-like peptide 1; DPP-4: Dipeptidyl peptidase-4.

were diagnosed aged 60 years or older. The age at which participants were diagnosed with T2D ranged from 18 to 74 years (Figure 1). Table 2 presents participant characteristics by diagnostic age categories. Participants who were diagnosed under 40 years had a median current age of 46 years (IQR: 38-55) at study entry compared to 71 years (IQR: 68-73) among participants diagnosed at 60 years or over. As expected, median T2D duration was highest among participants who were diagnosed under the age of 40 years (11 years, IQR: 5-21) and lowest in parti-cipants diagnosed at 60 years or over (5 years, IQR: 3-8 years).

A higher proportion of participants diagnosed under the age of 40 were female (49.0%) compared to those diagnosed between 40-59 years (33.2%) or over 60 years (33.8%). Although the most common ethnicity across all diagnostic age groups was white, there were proportionally more Asian participants in those diagnosed before the age of 40 (28.1%), compared to those diagnosed aged 40-59 years or aged 60 years or older (17.1% and 7.6%, respectively). Participants diagnosed under the age of 40 were also more likely to be current smokers (12.2%) and to have a family history of T2D (45.9%). The prevalence of all cardiovascular complications was lower at the point of study entry among participants diagnosed under the age of 40, compared to those diagnosed at the age of 40 or over. The prevalence of metabolic syndrome was higher among participants diagnosed under 40 years (94.1%) compared to those diagnosed between 40-59 years (90.2%) or at 60 years or over (85.6%). The proportion of participants using glucose-lowering medications was higher in participants diagnosed before 40 years (94.9%) compared to participants diagnosed between 40-59 years (89.5%) or 60 years or over (70.0%), whilst the opposite trend was observed for lipid-lowering or antihypertensive medications.

Cardiovascular risk factors

Table 3 displays participants' cardiovascular risk factor profiles by diagnostic age categories. Participants diagnosed with T2D under the age of 40 had a higher body weight (95.2 kg, IQR: 82.5-108.9 kg) compared to participants diagnosed between the ages of 40-59 years (92.0 kg, IQR: 79.6-105.6 kg) or at 60 years or over (84.6 kg, IQR: 73.7-97.4 kg). BMI was also highest among participants diagnosed under 40 years (33.0 kg/m^2, IQR: 29.0-36.8 kg/m^2) compared to those diagnosed between 40-59 years (31.6 kg/m^2, IQR: 28.0-35.3 kg/m^2) or those diagnosed later than 60 years of age (29.2 kg/m^2, IQR: 26.3-33.0 kg/m^2). A similar trend was observed for waist circumference and body fat percentage.

Median HbA1c was also higher among participants diagnosed under the age of 40 (7.5%, IQR: 6.7%-8.5%) compared to those diagnosed between the age of 40-59 years (7.1%, IQR: 6.4%-7.9%) or at 60 years or over (6.5%, IQR: 6.0%-7.2%). Additionally, a marginally more adverse lipid profile was identified among participants diagnosed under the age of 40, showing higher total cholesterol, LDL cholesterol and triglycerides, and lower HDL cholesterol compared to participants diagnosed at 40 years or over.

Table 2 Demographic characteristics, cardiovascular complications and medication use by age of diagnosis

	Age at T2DM diagnosis			Total sample (*n* = 1409)
	Under 40 yr (*n* = 196)	40-59 yr (*n* = 846)	60 yr or over (*n* = 367)	
Number of participants from each dataset, *n* (%)				
CODEC	111 (56.6)	636 (75.2)	326 (88.8)	1073 (76.2)
LYDIA	35 (17.9)	41 (4.9)	0 (0.0)	76 (5.4)
EXPEDITION	20 (10.2)	0 (0.0)	0 (0.0)	20 (1.4)
DIASTOLIC	17 (8.7)	72 (8.5)	0 (0.0)	89 (6.3)
PREDICT	13 (6.6)	97 (11.5)	41 (11.2)	151 (10.7)
Current age, yr (*n* = 1408)	46 (38-55)	61 (56-67)	71 (68-73)	63 (55-69)
Diabetes duration, yr (*n* = 1408)	11 (5-21)	10 (5-15)	5 (3-8)	8 (4-14)
Sex, *n* (%)				
Male	100 (51.0)	565 (66.8)	243 (66.2)	908 (64.4)
Female	96 (49.0)	281 (33.2)	124 (33.8)	501 (35.6)
Ethnicity, *n* (%)				
White	125 (63.8)	665 (78.6)	333 (90.7)	1123 (79.7)
Asian	55 (28.1)	145 (17.1)	28 (7.6)	228 (16.2)
Other	6 (3.1)	33 (3.9)	5 (1.4)	44 (3.1)
Unknown	10 (5.1)	3 (0.4)	1 (0.3)	14 (1.0)
Smoking status, *n* (%)				
Current smoker	24 (12.2)	66 (7.8)	20 (5.5)	110 (7.98)
Ex-smoker	58 (29.6)	367 (43.4)	182 (49.6)	607 (43.1)
Never smoked	114 (58.2)	413 (48.8)	165 (45.0)	692 (49.1)
Family history of T2D, *n* (%)				
Yes	90 (45.9)	326 (38.5)	131 (35.7)	547 (38.8)
No	37 (18.9)	264 (31.2)	169 (46.1)	470 (33.3)
Unknown	69 (35.2)	256 (30.3)	67 (18.3)	393 (27.9)
Cardiovascular complications, *n* (%)				
Myocardial infarction (*n* = 1233)	7 (4.3)	60 (8.0)	36 (11.1)	103 (8.4)
Heart failure (*n* = 1229)	4 (2.5)	12 (1.6)	9 (2.8)	25 (2.0)
Heart valve disease (*n* = 1228)	3 (1.8)	22 (3.0)	11 (3.4)	36 (2.9)
Atrial fibrillation (*n* = 1223)	2 (1.2)	42 (5.7)	20 (6.2)	64 (5.2)
Peripheral vascular disease (*n* = 1227)	7 (4.4)	43 (5.8)	21 (6.5)	71 (5.8)
Stroke (*n* = 1235)	3 (1.9)	31 (4.1)	24 (7.4)	58 (4.7)
Angina (*n* = 1230)	5 (3.1)	60 (8.1)	33 (10.2)	98 (8.0)
Glucose-lowering medication use, *n* (%)				
Any glucose-lowering medication (*n* = 1403)	185 (94.9)	753 (89.5)	257 (70.0)	1195 (85.2)
Insulin	74 (37.8)	178 (21.0)	24 (6.5)	276 (19.6)
Metformin (*n* = 1407)	157 (80.1)	658 (78.0)	234 (63.8)	1049 (74.6)
Sulphonylurias (*n* = 1407)	36 (18.4)	205 (24.3)	51 (13.9)	292 (20.8)
DPP-4 inhibitors	18 (9.2)	139 (16.4)	39 (10.6)	196 (13.9)
GLP-1 agonists	33 (16.8)	63 (7.5)	9 (2.5)	105 (7.5)
SGLT2 inhibitors (*n* = 1389)	27 (17.0)	89 (11.5)	15 (4.1)	131 (10.1)

Other[1] (*n* = 1390)	3 (1.9)	19 (2.4)	4 (1.1)	26 (2.0)
Lipid-lowering medication use, *n* (%)				
Any lipid-lowering medication (*n* = 1407)	112 (57.4)	583 (69.0)	254 (69.2)	949 (67.5)
Statins (*n* = 1408)	108 (55.4)	580 (68.6)	251 (68.4)	939 (66.7)
Fibrates (*n* = 1407)	10 (5.1)	23 (2.7)	4 (1.1)	37 (2.6)
Antihypertensive medication use, *n* (%)				
Any antihypertensive medication (*n* = 1389)	97 (54.8)	582 (68.8)	252 (68.9)	931 (67.0)
ACE inhibitors (*n* = 1390)	68 (38.4)	356 (42.1)	125 (34.1)	549 (39.5)
Alpha blockers (*n* = 1388)	8 (4.6)	86 (10.2)	53 (14.5)	147 (10.6)
Angiotensin receptor blockers (*n* = 1389)	17 (9.7)	134 (15.8)	61 (16.6)	212 (15.3)
Beta blockers (*n* = 1388)	20 (11.4)	157 (18.6)	70 (19.1)	247 (17.8)
Calcium channel blockers (*n* = 1389)	31 (17.6)	246 (29.1)	103 (28.1)	380 (27.4)
Diuretics (*n* = 1389)	25 (14.2)	122 (14.4)	58 (15.8)	205 (14.8)
Metabolic syndrome prevalence, *n* (%)[2] (*n* = 1290)	159 (94.1)	697 (90.2)	298 (85.6)	1154 (89.5)

[1]Includes alpha glucosidase inhibitors, thiazolidinediones and meglitinides.
[2]Defined using the global definition published by Alberti *et al*[24] (2005).
Data presented as median or frequency (%), as appropriate. T2DM: Type 2 diabetes mellitus; SGLT-2: Sodium-glucose cotransporter-2; GLP-1: Glucagon-like peptide 1; DPP-4: Dipeptidyl peptidase-4.

Table 3 Cardiovascular risk factors by age at diagnosis

	Age at T2DM diagnosis			Total sample (*n* = 1409)
	Under 40 yr (*n* = 196)	40-59 yr (*n* = 846)	60 yr or over (*n* = 367)	
Weight (kg)	95.2 (82.5-108.9)	92.0 (79.6-105.6)	84.6 (73.7-97.4)	90.6 (78.2-103.8)
BMI (kg/m^2)	33.0 (29.0-36.8)	31.6 (28.0-35.3)	29.2 (26.3-33.0)	31.1 (27.5-35.0)
Waist circumference (cm), *n* = 1403	112.0 (102.2-119.1)	109.0 (100.0-118.0)	104.0 (96.8-113.5)	108.0 (99.0-117.8)
Body fat (%), *n* = 1076	36.9 (29.5-44.5)	34.0 (27.6-40.7)	32.5 (26.3-41.0)	33.8 (27.4-41.2)
HbA1c (%), *n* = 1370	7.5 (6.7-8.5)	7.1 (6.4-7.9)	6.5 (6.0-7.2)	7.0 (6.3-7.8)
Total cholesterol (mmol/L), *n* = 1352	4.4 (3.8-5.2)	4.2 (3.6-4.9)	4.1 (3.5-4.8)	4.2 (3.6-4.9)
LDL cholesterol (mmol/L), *n* = 1308	2.3 (1.8-2.9)	2.1 (1.6-2.7)	2.1 (1.6-2.6)	2.1 (1.6-2.7)
HDL cholesterol (mmol/L), *n* = 1338	1.1 (1.0-1.4)	1.2 (1.0-1.5)	1.3 (1.1-1.5)	1.2 (1.0-1.5)
Triglycerides (mmol/L), *n* = 1351	1.8 (1.2-2.7)	1.7 (1.2-2.3)	1.5 (1.1-2.1)	1.6 (1.1-2.3)
Systolic blood pressure (mmHg), *n* = 1408	128.0 (119.0-140.0)	135.0 (124.0-146.5)	137.0 (125.5-148.7)	135.0 (123.8-146.0)
Diastolic blood pressure (mmHg), *n* = 1408	83.0 (76.5-90.5)	82.0 (76.0-89.0)	79.5 (73.0-86.5)	81.5 (75.0-88.6)

Data presented as median. LDL: Low-density lipoprotein; HDL: High-density lipoprotein; BMI: Body mass index; T2DM: Type 2 diabetes mellitus.

Model results

As shown in Table 4, younger age at diagnosis of T2D was significantly associated with higher body weight, BMI, waist circumference and HbA1c. Results from Model 3 (adjusted for duration of T2D, sex, ethnicity and smoking status) showed that each one year reduction in age at diagnosis of T2D was significantly associated with 0.67 kg [95% confidence interval (CI): 0.52-0.82 kg] higher body weight, 0.18 kg/m^2 (95%CI: 0.10-0.25 kg/m^2) higher BMI, 0.32 cm (95%CI: 0.14-0.49 cm) higher waist circumference and 0.03% (95%CI: 0.03%-0.04%) higher HbA1c. Similarly, results from Model 3 indicate that each one year reduction in age at diagnosis was significantly associated with 0.01 mmol/L (95%CI: 0.01-0.02 mmol/L) higher total cholesterol, 0.01 mmol/L higher LDL cholesterol (95%CI: 0.01-0.02 mmol/L) and 0.02 mmol/L (95%CI: 0.01-0.03 mmol/L) higher triglycerides, after adjustment for the same covariates. Each one year reduction in diagnostic age was significantly associated with 0.22 mmHg

Table 4 Results from linear regression models investigating the effect of age at diagnosis on each cardiovascular risk factor

	Model 1		Model 2		Model 3	
	Estimate	*n*	Estimate	*n*	Estimate	*n*
Weight (kg)	-0.32 [(-0.51) to (-0.14)][a]	1409	-0.45 [(-0.60) to (-0.31)][a]	1408	-0.67 [(-0.82) to (-0.52)][a]	1394
BMI (kg/m^2)	-0.11 [(-0.19) to (-0.02)][b]	1409	-0.15 [(-0.23) to (-0.07)][a]	1408	-0.18 [(-0.25) to (-0.10)][a]	1394
Waist circumference (cm)	-0.21 [(-0.32) to (-0.09)][a]	1403	-0.23 [(-0.41) to (-0.05)][b]	1402	-0.32 [(-0.49) to (-0.14)][a]	1388
Body fat (%)	-0.10 (-0.80 to 0.60)	1076	-0.16 (-0.85 to 0.53)	1075	-0.11 (-0.43 to 0.20)	1073
HbA1c (%)	-0.04 [(-0.04) to (-0.03)][a]	1370	-0.03 [(-0.04) to (-0.03)][a]	1369	-0.03 [(-0.04) to (-0.03)][a]	1356
Total cholesterol (mmol/L)	-0.01 (-0.01 to 0.00)	1352	-0.02 [(-0.02) to (-0.01)][a]	1351	-0.01 [(-0.02) to (-0.01)][a]	1337
LDL cholesterol (mmol/L)	-0.01 (-0.01 to 0.00)[b]	1308	-0.01 [(-0.02) to (-0.01)][a]	1307	-0.01 [(-0.02) to (-0.01)][a]	1294
HDL cholesterol (mmol/L)	0.00 (0.00 to 0.01)	1338	0.00 (0.00 to 0.01)	1337	0.01 (0.00 to 0.01)	1323
Triglycerides (mmol/L)	-0.01 (-0.03 to 0.00)	1351	-0.02 [(-0.03) to (-0.01)][b]	1350	-0.02 [(-0.03) to (-0.01)][a]	1336
Systolic BP (mmHg)	0.24 (0.14 to 0.35)[a]	1408	0.31 (0.11 to 0.51)[b]	1407	0.24 (0.02 to 0.45)[b]	1393
Diastolic BP (mmHg)	-0.10 (-0.21 to 0.02)	1408	-0.20 [(-0.29) to (-0.12)][a]	1407	-0.22 [(-0.30) to (-0.14)][a]	1393

[a]$P < 0.01$.
[b]$P < 0.05$.
Data presented as coefficient. Model 1: Unadjusted univariable model, Model 2: Adjusted for duration of T2D, Model 3: Adjusted for duration of T2D, sex, ethnicity and smoking status. LDL: Low-density lipoprotein; HDL: High-density lipoprotein; BMI: Body mass index; T2DM: Type 2 diabetes mellitus; HbA1c: Glycaemic control; BP: Blood pressure.

(95%CI: 0.14-0.30 mmHg) higher diastolic blood pressure, but 0.24 mmHg (95%CI: 0.02-0.45 mmHg) lower systolic blood pressure.

DISCUSSION

This analysis investigated the association between age at diagnosis of T2D and the cardiovascular risk profile among 1410 adults with T2D, using a diverse pooled dataset. The demographic characteristics of the participants included in our analysis varied by diagnostic age, with a higher proportion of females and people of Asian ethnicity among participants diagnosed earlier in life. These results are consistent with previous studies, which have also highlighted increased risk of microvascular and macrovascular complications, as well as incidence of certain co-morbidities, in these high risk subgroups[2,6,15-17]. In our analysis, younger age at diagnosis was also significantly associated with higher BMI, supporting findings from previous studies[3,7,9]. However, the current analysis adds to previous findings by quantifying the change in several cardiovascular risk factors, including body weight, BMI, waist circumference, HbA1c, lipids and blood pressure, for each year earlier diagnosis of T2D.

The association between younger age at diagnosis of T2D and poorer HbA1c identified in this analysis is supported by findings from previous studies[3,6-9]. One study from Asia investigated the association between HbA1c and age of diagnosis, analyzed as a continuous variable, reporting that each one year earlier age at diagnosis was significantly associated with 0.01% higher HbA1c. This is similar, albeit smaller in magnitude, to the results of our current analysis, which identified a 0.03% increase in HbA1c for each year earlier diagnosis.

In the current analysis, diagnosis of T2D at a younger age was significantly associated with higher total and LDL cholesterol and higher triglycerides, however no significant association was observed between age at diagnosis and HDL cholesterol. Similarly, previous studies have reported conflicting results for the relationship between age at diagnosis and lipid profile. For example, Yeung *et al*[3] reported a significant association between age at diagnosis and all lipid markers, whereas Huang *et al*[7] found participants with early-onset T2D had significantly higher total cholesterol and triglycerides compared to those with later-onset T2D, whilst no significant differences were observed from LDL or HDL cholesterol. Younger age at T2D diagnosis was significantly associated with higher diastolic blood pressure and lower systolic blood pressure in this analysis, which is also consistent with previous studies[3,8,9].

The adverse risk factor phenotype observed among younger adults with T2D may contribute to their significantly increased relative risk of microvascular and macrovascular disease and mortality. A recent meta-analysis of 26 studies found that for every one year increase in age at diagnosis, the risk of microvascular disease, macrovascular disease and all-cause mortality fell by 5%, 3% and 4%,

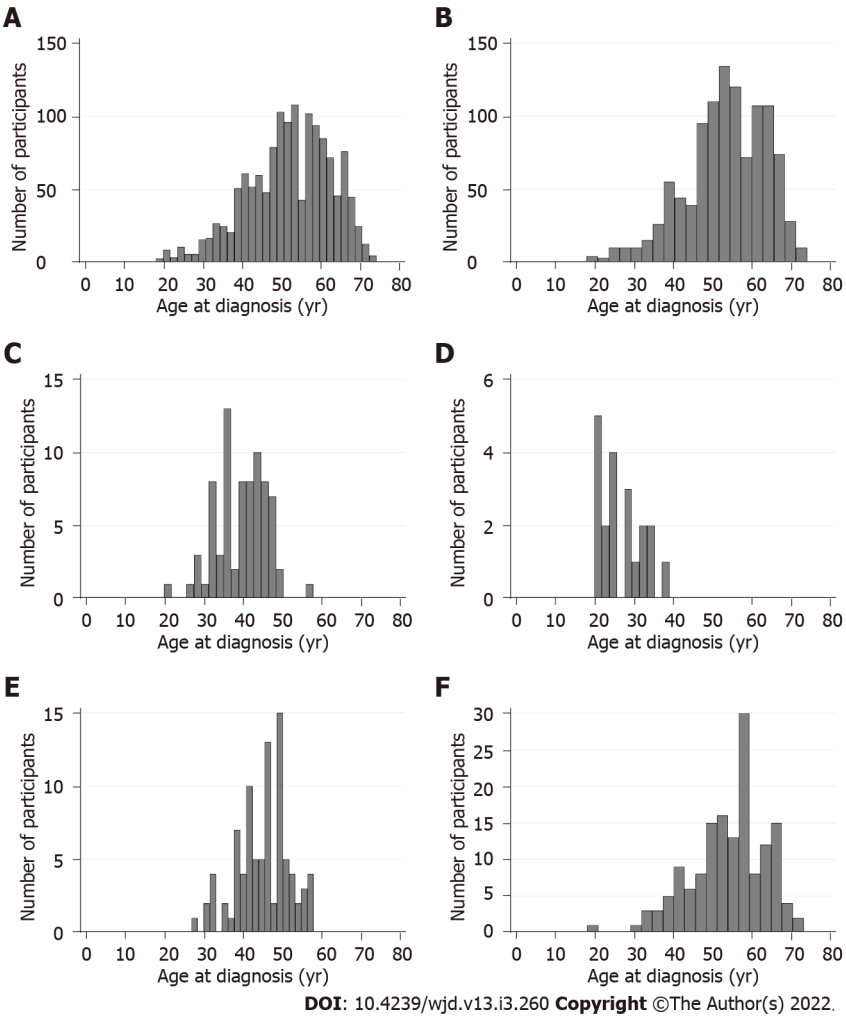

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Figure 1 Frequency distribution of age at diagnosis from each study. A: All participants; B: CODEC participants; C: LYDIA participants; D: EXPEDITION participants; E: DIASTOLIC participants; F: PREDICT participants.

respectively[5]. Research has also shown that the risk of cardiovascular complications and all-cause mortality can be reduced by the control of multiple cardiovascular risk factors, even among younger adults[18-20]. It is therefore imperative that adults with early-onset T2D have access to fit-for-purpose, multifactorial interventions to prevent long term complications, particularly given the global increase in the prevalence of early-onset T2D, and evidence that less than half of younger adults with T2D meet HbA1c targets[21]. Such interventions must also be tailored specifically to the co-occurring challenges that adults with early-onset T2D may face (*e.g.*, early careers, ongoing education and young families), which may be different to older adults, and also allow for sex and cultural differences between individuals. Work to guide the development of such tailored approaches is urgently required, particularly given that adults with early-onset T2D are underrepresented in existing T2D trials[2,22].

This analysis has many strengths. Firstly, the pooled dataset used included a large sample of adults diagnosed with T2D over a wide age range (18-74 years of age) increasing the reliability and generalisability of the conclusions made. The use of age at diagnosis as a continuous variable in the analysis is another benefit, given that most previous literature has investigated age at diagnosis as a categorical variable, comparing people with 'early-onset' T2D to those with 'later-onset' T2D. Although such studies are valuable in assessing whether adults classified as 'early onset' have more cardiovascular risk factors, the range of ages included in the 'early-onset' and 'late-onset' categories are very wide and therefore it was previously unknown how diagnostic age was associated with cardiovascular risk factors within each of these categories. The current study has provided such insight by showing that each reduction of one year in age at diagnosis was significantly associated with a more adverse adiposity, HbA1c, and lipid profile, even after the adjustment for disease duration.

Limitations of the study must also be noted. As the information relating to diagnostic age was self-reported, some participants may not have accurately recalled the date at which they were diagnosed. However, there is evidence that self-reported age at diagnosis of T2D is fairly accurate and a valid measure of diagnostic age[23]. There is also the possibility of selection bias as the data used in this analysis is from volunteers who were motivated to undertake the research studies. In addition, it is

possible that differences in recruitment rates by age at diagnosis and investigated risk factors acted to introduce a form of collider bias. Furthermore, only variables collected in the studies were available for adjustment in this analysis, therefore the effects of other covariates could not be assessed. As the cardiovascular risk factors investigated in this analysis were collected at study enrolment rather than at diagnosis of T2D, the results from this analysis do not indicate how the cardiovascular risk profile differs by diagnostic age at the time of diagnosis. Lastly, the age at diagnosis, age at enrolment and duration of diabetes are correlated and therefore disentangling the effect of one from the others is complex. Nevertheless, the results from the current analysis were unaffected by adjustment for diabetes duration.

CONCLUSION

In conclusion, this study supports previous literature demonstrating an association between younger diagnosis of T2D and a more adverse cardiovascular risk profile. This highlights the need for interventions targeting multiple risk factors in younger adults with T2D in order to reduce their risk of cardiovascular complications and mortality.

ARTICLE HIGHLIGHTS

Research background

The prevalence of type 2 diabetes (T2D) among younger adults is increasing, and is associated with a higher relative risk of mortality and diabetes-related complications compared to older adults with T2D. This may be due to younger adults with T2D having a more adverse cardiovascular risk factor profile.

Research motivation

Although some research has observed a more adverse cardiovascular risk profile among younger adults with T2D, conflicting findings and methodological limitations have emerged within these studies.

Research objectives

To use a pooled dataset to investigate the association between age at diagnosis (as a continuous variable) and the cardiovascular risk factor profile of adults with T2D.

Research methods

The pooled dataset used for this analysis included 1409 participants, 196 of whom were diagnosed with T2D under the age of 40 years. Descriptive analysis and both univariable and multivariable linear regression models were used to investigate the association between diagnostic age and cardiovascular risk factors [weight, body mass index (BMI), waist circumference, body fat percentage, glycaemic control (HbA1c), lipid profile and blood pressure].

Research results

Results from the analysis revealed that younger age at T2D diagnosis was significantly associated with higher weight, BMI, waist circumference, HbA1c and a more adverse lipid profile, even once confounding factors such as diabetes duration, sex and ethnicity were accounted for.

Research conclusions

This analysis supports previous studies which demonstrate an association between younger age at T2D diagnosis and a worse cardiovascular risk factor profile.

Research perspectives

The results from this analysis highlight the importance of multifactorial interventions targeting multiple risk factors in younger adults with T2D, in order to reduce their risk of mortality and cardiovascular complications.

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FOOTNOTES

Author contributions: Davies MJ and Sargeant JA generated the study idea; Barker MM, Zaccardi F, Henson J, Yates T and Sargeant JA prepared and conducted the analysis; Barker MM, Zaccardi F, Davies MJ and Sargeant JA interpreted the analysis and drafted the manuscript, with clinical and/or academic input from co-authors; all authors reviewed and approved the final manuscript.

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Institutional review board statement: All studies included in the pooled dataset used for this analysis gained full ethical approval (CODEC: 16/WM/0457; EXPEDITION: 08/H0407/8; LYDIA: 13/WM/0311; DIASTOLIC: 15/WM/0222; PREDICT: 17/WM/0192).

Informed consent statement: All participants included in the studies provided written informed consent.

Conflict-of-interest statement: Barker MM, Zaccardi F, Brady EM, Gulsin GS, Hall AP, Henson J, Htike ZZ, McCann GP, Redman EL, Webb DR and Yeo J report no conflicts of interest. Khunti K has acted as consultant, advisory board member and speaker for Abbott, Amgen, Astrazeneca, Bayer, NAPP, Lilly, Merck Sharp and Dohme, Novartis, Novo Nordisk, Roche, Berlin-Chemie AG/Menarini Group, Sanofi-Aventis, Servier, Boehringer Ingelheim, EACME grants from Boehringer Ingelheim, AstraZeneca, Novartis, Novo Nordisk, Sanofi-Aventis, Lilly, Merck Sharp & Dohme. Yates T and Sargeant JA are supported by the NIHR Leicester BRC and have received project funding in the form an investigator-initiated grant from AstraZeneca. EGW has received personal fees from Abbott Diabetes Care, Dexcom, Eli lilly, Insulet, Medtronic, Novo Nordisk, Sanofi Aventis. Davies MJ has acted as consultant, advisory board member and speaker for Novo Nordisk, Sanofi, Lilly and Boehringer Ingelheim, an advisory board member and speaker for AstraZeneca, an advisory board member for Janssen, Lexicon, Servier and Gilead Sciences Ltd and as a speaker for Napp Pharmaceuticals, Mitsubishi Tanabe Pharma Corporation and Takeda Pharmaceuticals International Inc. She has received grants in support of investigator and investigator initiated trials from Novo Nordisk, Sanofi-Aventis, Lilly, Boehringer Ingelheim, AstraZeneca and Janssen.

Data sharing statement: Data included in this pooled analysis will be made available, after publication, to anyone upon reasonable request to the corresponding author.

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LETTER TO THE EDITOR

Sodium-glucose co-transporter 2 inhibitors induced euglycemic diabetic ketoacidosis within four days of initiation

Almurtada Razok, Fateen Ata, Sara Mohamed Ibrahim Ahmed, Dabia Hamad S H Al Mohanadi

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Abstract

Euglycemic diabetic ketoacidosis (EDKA) is a well-known complication of sodium-glucose co-transporter 2 inhibitors, and many cases with variable onset following the initiation of these agents are reported before, with a median onset of approximately 2 wk. This letter discusses a 45-year-old lady who initially presented with ischemic stroke but developed EDKA 4 d after starting empagliflozin, a rare occurrence. The patient had severe metabolic acidosis that necessitated admission into the intensive care unit. Prompt discontinuation of empagliflozin and DKA management resulted in clinical recovery.

Key Words: Euglycemic diabetic ketoacidosis; Sodium-glucose co-transporter 2 inhibitors; Type 2 diabetes mellitus; Empagliflozin

Core Tip: With a steady surge in the prescription of sodium-glucose co-transporter 2 inhibitors (SGLT2-i) in medical conditions including type 2 diabetes mellitus (DM), type 1 DM, and heart failure, there are increasingly reported cases of euglycemic diabetic ketoacidosis (EDKA) with their use. EDKA in the context of SGLT2-i use is reported in various patients with different precipitating factors, some even with no inciting event. One of the rarely reported inciting events is stroke. Another aspect of SGLT2-i induced EDKA which remains relatively less understood is the time of initiation of the drug to the development of EDKA. In our patient, severe EDKA developed within 4 d of empagliflozin initiation, necessitating intensive care and discontinuation of empagliflozin, resulting in complete recovery regarding the EDKA.

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TO THE EDITOR

With great interest, we read the recent article "Euglycemic diabetic ketoacidosis: A missed diagnosis" by Nasa *et al*[1]. The authors have described various factors that can potentiate euglycemic diabetic ketoacidosis (EDKA) in sodium-glucose co-transporter 2 inhibitor (SGLT2-i) use. There is a steady increase in EDKA reports secondary to SGLT2-i. Most of the articles mention a precipitating factor behind the development of EDKA in patients taking SGLT2-i. More extensive studies mention no or unknown precipitating factor in 16%-51% of cases[2,3]. This creates a need to explore a possible direct link of SGLT2-i in the development of EDKA in an otherwise healthy patient with diabetes. Acute vascular events such as stroke are infrequent inciting events for EDKA in the setting of SGLT2-i use.

We recently encountered an interesting case of a patient with type 2 diabetes mellitus (T2DM) who was admitted with acute stroke and developed EDKA within 4 d of initiation of empagliflozin.

A 45-year-old woman presented with sudden onset left arm weakness and slurred speech with facial droop. Magnetic resonance imaging revealed a right basal ganglia acute infarction, in addition to left parietal subcortical microangiopathic changes. The patient had a history of breast carcinoma, treated with mastectomy and maintenance tamoxifen. She also had T2DM and was prescribed sitagliptin/metformin 50/1000 mg two tablets daily. However, the patient was non-compliant with the medication and was not checking her blood sugar regularly.

The patient was started on dual antiplatelet therapy (aspirin 100 mg and clopidogrel 75 mg once daily) after establishing the diagnosis of an acute stroke. Her HbA1c was 14%, confirming a poor control of her diabetes. To manage her poorly controlled diabetes mellitus, sitagliptin/metformin was continued, with the addition of insulin glargine 12 units at bedtime and empagliflozin 10 mg once daily.

Four days later, the patient developed vomiting and generalized fatigue. Arterial blood gas showed severe metabolic acidosis with a pH of 6.9 and bicarbonate level of 3 mEq/L (reference range: 22-26 mEq/L). Serum B-hydroxy butyrate was higher than the reported threshold of 9.60 mmol/L (reference range: 0.03-0.3). Her blood glucose level at the time was 10.3 mmol/L (reference range: 3.3-5.5), and her urine dipstick showed +4 ketone. She was diagnosed with severe EDKA and was shifted to the medical intensive care unit for further management and treatment.

Regular insulin infusion and intravenous fluids were initiated, and a right internal jugular line was inserted for monitoring and resuscitation. Her arterial blood gas was measured every 2 h, and serum ketones were measured daily. Forty-eight hours later, the patient's condition improved, and she started tolerating oral feed. Her ABG results showed significant improvement with the closure of the anion gap (Table 1). She was started on subcutaneous glargine 20 units daily and insulin as part 7 units three times a day.

The patient was consequently shifted back to the care of the general medicine team, where her glycemic control was monitored closely. After ensuring the patient's fitness and stability, she was transferred to a physical and occupational therapy rehabilitation facility.

Our case highlights that in the presence of a precipitating factor, SGLT2-i drugs can cause an early and severe EDKA. We recommend that wherever other choices are available, initiation of SGLT2-i should be delayed until patients are otherwise healthy and not admitted with an acute event. SGLT2-i medications should ideally be started in an outpatient setting, and the patients should be counseled not to rely on blood glucose and seek immediate medical attention when experiencing symptoms of DKA.

Table 1 Laboratory investigations of the patient during euglycemic diabetic ketoacidosis

Investigation	Onset	24 h	48 h	Reference
PH	6.9	7.32	7.42	7.35-7.45
HCO_3 (mmol/L)	3	12.5	20.6	22-29
Glucose (mmol/L)	10.3	10.8	6.4	3.3-5.5
Sodium (mmol/L)	140	137	139	133-146
Potassium (mmol/L)	3.8	3.2	3.4	3.5-5.3
Chloride (mmol/L)	101	111	110	95-108
Anion Gap	36	13.5	8.4	10-12
Lactate (mmol/L)	1.3	0.7	0.9	0.36-1.6
B-hydroxybutyrate (mmol/L)	> 9.60	1.22	0.11	0.03-0.3

FOOTNOTES

Author contributions: Razok A, Ata F, and Ahmed SMI wrote the letter; Al Mohanadi DHSH revised the letter.

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LETTER TO THE EDITOR

Free fatty acids, glucose, and insulin in type 2 diabetes mellitus

Rob NM Weijers

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Abstract

Xu *et al* used the HOMA2 model to estimate the β-cell function and insulin resistance levels in an individual from simultaneously measured fasting plasma glucose and fasting plasma insulin levels. This method is based on the assumption that the glucose-insulin axis is central for the metabolic activities, which led to type 2 diabetes. However, significant downregulation of both the *NKX2-1* gene and the *TPD52L3* gene force an increase in the release of free fatty acids (FFAs) into the blood circulation, which leads to a marked reduction in membrane flexibility. These data favor a FFA-glucose-insulin axis. The authors are invited to extend their study with the introduction of the saturation index (number of carbon-carbon double bonds per 100 fatty-acyl chains), as observed in erythrocytes.

Key Words: Free fatty acids; Membrane flexibility; *NKX2-1* gene; RNA sequencing; Type 2 diabetes; *TPD52L-3* gene; Unsaturation index

Core Tip: A substantial reduction in both NKX2-1 and TPD52L3 proteins is largely responsible for a reduction in carbon-carbon double bonds of phospholipids which, in turn, translates into the redistribution of the lateral pressure profile, and thereby reduces the transport speed of glucose molecules across the cell membrane. Consequently, the amount of plasma glucose entering the β-cell *via* GLUT2 gives a false negative result. Also the redistribution of the lateral pressure profile lowers the insulin release from β-cells into the blood circulation. Both phenomena cause the onset of type 2 diabetes mellitus.

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TO THE EDITOR

In the August 2021 issue of *World J Diabetes*, Xu *et al*[1] reported on the association of β-cell function and insulin resistance with pediatric type 2 diabetes among Chinese children. The term "insulin resistance" in the article needs additional clarification and review.

As early as 1933, there was as yet no general agreement as to the definition of the term "insulin resistance" and thus gaps in research and clinical care persisted. The breakthrough of the correct description of the term was a clear example of serendipity. A study by Takematsu *et al*[2] compared genome-wide changes in the gene expression in skin between patients with type 2 diabetes and non-diabetic patients *via* RNA sequencing, resulting in the identification of 64 significantly upregulated genes and 120 significantly downregulated genes. Among these regulated genes, the most downregulated gene was *NKX2-1*, with a down regulation value of 3.7×10^{-9}, and in the metabolism category the most downregulated gene was *TPD52L3*, also with a down regulation value of 3.7×10^{-9}. The latter gene has not been linked to type 2 diabetes.

Defective NKX2-1 production is associated with an essential reduction in the activity of the mitochondrial respiratory chain complex, which reduces ATP production. This idea is supported by the data from a study suggesting that a dysregulation of intramyocellular fatty acid metabolism in the offspring of patients with type 2 diabetes was associated with an inherited defect in mitochondrial oxidative phosphorylation[3]. To restore ATP production, the β-oxidation of fatty acids provides assistance by increasing the levels of plasma free fatty acids (FFAs) *via* hydrolysis. Calculation of the saturation indices (number of cis carbon-carbon double bonds per 100 fatty acyl-chains[4]) of FFAs released from human white fat cells and human plasma FFAs in healthy controls reveals that the index of the former is substantially lower (85.5 and 191.9, respectively; $\Delta = 55.4\%$)[5]. Thus, we can conclude that an increase in the release of FFAs into the blood circulation due to an essential reduction in the activity of the mitochondrial respiratory chain complex leads to a marked reduction in the unsaturated index.

In a previous study, the author found that, in brown adipose tissue, the mitochondrial population exists as two subclasses: cytoplasmic mitochondria that do not adhere to lipid droplets and mitochondria that do adhere to lipid droplets. The lipid droplets are cytosolic storage organelles consisting mostly of neutral lipids and enclosed by a phospholipid monolayer membrane[6]. This monolayer has persistent surface packing defects, whereby neutral lipids are accessible to the aqueous cytoplasm and the blood circulation. The idea is that TPD52L3 covers these defects in healthy individuals. Thus, it seems likely that the significant downregulation of *TPD52L3* causes an increase in FFAs in the blood circulation and also lowers the saturation index.

Thus, we can conclude that the downregulation of *NKX2-1* and *TPD52L3* forces an increase in the release of FFAs into the blood circulation due to the leaky lipid droplets and the essential reduction in mitochondrial oxidative and phosphorylation activity, and thereby reduces the unsaturation index, as demonstrated in impaired glucose tolerance[7] (Table 5 in Weijers[7]), gestational diabetes mellitus[7] (Table 5 in Weijers[7]), and type 2 diabetes[4] (Table 2 in Weijers[4]). These phenomena lead to a marked shift from unsaturated to saturated acyl chains in the membrane phospholipids, which redistributes the lateral pressure profile of the cell membrane[8]. The redistribution of this profile narrows the pore diameter of the transmembrane glucose transport channels of all class I glucose transporter proteins, and thus reduces the rate of transport of glucose molecules across the cell membrane, initiating the onset of type 2 diabetes[7].

The following conclusions can be drawn from the presented information. First, type 2 diabetes is characterized by reduced membrane flexibility in the pancreatic β-cell, which adversely affects the amount of glucose entering the β-cell *via* GLUT2, and thereby lowers the synthesis of the necessary amount of circulating insulin molecules. Secondly, fusion of the insulin-containing granule with the β-cell plasma membrane, followed by the formation of a suitable pore diameter for insulin transport into the blood circulation, requires high flexibility in both the granule-cell membrane and the β-cell membrane. The reduction in the flexibility of both membranes lowers the insulin release from the β-cell into the blood circulation. These facts underline the fact that a reduction in membrane flexibility lowers not only the rate of transport of glucose molecules into the β-cell but also the rate of transport of insulin molecules from the β-cell into the blood circulation.

Up to now, it has been thought that the glucose-insulin axis is central to the metabolic activities that lead to type 2 diabetes. A publication in 1992 is an exception in this respect, having the title: "What if Minkowski had been ageusic? An alternative angle on diabetes"[9]. This study suggested that the basic pathophysiological mechanisms of type 2 diabetes might be more readily understood if viewed in the context of underlying abnormalities of lipid metabolism. Xu *et al*[1] used the HOMA2 model to estimate β-cell function and insulin resistance levels in a pediatric individual from simultaneously measured fasting serum glucose and fasting serum insulin concentrations. The summarized phenomena, in my opinion, are a scientific basis for the idea that membrane flexibility plays an important part in the onset of type 2 diabetes.

Therefore, I suggest that Xu *et al*[1] add to their article a follow-up study including the unsaturation index, as a parameter for membrane flexibility, based on the erythrocyte membrane fatty-acid compositions because, at the most basic level, the basal metabolic rate of a cell is directly linked to its cell

membrane's acyl composition[10]. A strong argument in favor of the FFA-glucose-insulin axis is the observation that in persons at high risk for type 2 diabetes, the incidence of diabetes was reduced by 58% with lifestyle intervention and by 31% with metformin, as compared with placebo[11]. It seems likely that physical activity, after all, raises the levels of the unsaturation index, in contrast to metformin.

FOOTNOTES

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LETTER TO THE EDITOR

Beyond diabetes remission a step further: Post bariatric surgery hypoglycemia

Devraj Lath, Kripa Elizabeth Cherian, Thomas Vizhalil Paul, Nitin Kapoor

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Abstract

Postbariatric hypoglycemia is a rare but increasingly recognized complication of bariatric surgery, with significant associated morbidity, and many patients often require multimodal treatment. A mixed meal challenge test is often helpful to diagnose this condition. This manuscript highlights the underlying mechanisms that lead to this condition and the novel emerging therapeutic targets that target these mechanisms.

Key Words: Postbariatric hypoglycemia; Hyperinsulinemic hypoglycemia; Avexitide; GLP-1 antagonist; Obesity

Core Tip: Postbariatric hypoglycemia is an uncommon complication presenting months to years after bariatric surgery (mostly in Roux-en-Y gastric bypasses) as postprandial hyperinsulinemic hypoglycaemia occurring 1-3 h after meals, and the associated neuroglycopenic symptoms can be incapacitating. Medical nutrition therapy forms the foundation of management, with pharmacotherapy and surgical interventions available for those who do not respond. An increased understanding of the implicated mechanisms has led to the development of targeted agents like avexitide, which has demonstrated good efficacy in a Phase 2 clinical trial (PREVENT) recently.

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TO THE EDITOR

We read with interest the review by Jin *et al*[1], who discussed the potential mechanisms underlying the remarkable efficacy of bariatric surgery in inducing remission of type 2 diabetes mellitus, ranging from 33% in adjustable gastric banding to up to 95% in biliopancreatic diversion. In a recent meta-analysis involving 174772 patients compared in 16 cohort studies and 1 controlled trial, bariatric surgery was associated with a reduction in the risk of all-cause mortality by 49.2% and an increased median life expectancy of 6.1 years. These benefits were even greater among those diagnosed with type 2 diabetes mellitus[2].

Postbariatric hypoglycaemia (PBH) is an infrequent but potentially debilitating complication, with a multicenter registry-based study in Spain having reported 22 patients developing hypoglycemia following 4645 interventions, amounting to an incidence rate of 0.47%[3]. In another study using registry data, 5040 Swedish patients that underwent Roux-en-Y gastric bypass (RYGB) were matched with 10 non-surgical controls each, with no preoperative difference in the frequency of hypoglycemia or potentially related diagnoses such as confusion, seizures or syncope[4]. Following gastric bypass, 0.2% of the post-gastric bypass cohort were admitted for hypoglycemia *vs* 0.04% of the general population. Although the overall incidence is variable, these patients were at a two- to sevenfold increased risk of hypoglycemia and related diagnoses when compared to their controls. The authors also found that there was no significant increase in the risk of postbariatric hypoglycemia or related diagnoses among patients undergoing restrictive procedures, namely vertical banded gastroplasty (4366) and gastric banding (2917) when matched with controls. Patients without diabetes especially are at an increased risk of hypoglycemia following bariatric surgery *vs* those managed medically[5]. Greater frequencies of hypoglycemia (32.6% and 22.6%) are observed in gastric bypass (GBP) and sleeve gastrectomy patients subjected to a two-hour oral glucose tolerance test (OGTT)[6], with much lower rates (2.3%)[6] reported in those undergoing gastric banding[5-6]. In another study by Tzovaras *et al*[7], 29% experienced definite dumping syndrome while another 16% had symptoms suggestive of the same.

Patients undergoing bariatric surgery (especially RYGB surgery) may develop severe vasomotor symptoms of sweating, dizziness, weakness and flushing, referred to as dumping syndrome. These are attributed to the osmotic effect of rapid food entry into the intestines, release of peptide hormones like vasoactive intestinal peptide, incretins and the enteric neural response. By contrast, the development of symptoms such as confusion, decreased vision, syncope, hunger, behavioural changes, syncope and seizures are suggestive of neuroglycopenia, and these patients are found to have low plasma glucose levels 1-3 h after a meal consistent with reactive hypoglycemia[8]. This occurs months to years after bariatric surgery, and though these phenomena have been classified as early and late dumping syndrome respectively[8], some suggest the term postbariatic hypoglycemia be used instead[8-9] and that the term dumping syndrome be reserved for the vasomotor symptoms caused by rapid gastric emptying, diagnosed by an increase in pulse rate > 10/min and/or a rise in hematocrit by 3% after an OGTT. Apart from the risks of severe hypoglycemia, these patients are also more likely to regain weight due to frequent food intake.

The diagnosis of hypoglycaemia requires the documentation of low plasma glucose during the presence of symptoms and/or signs attributable to hypoglycemia, which are relieved by raising the plasma glucose concentration (known as Whipple's triad)[10]. Postbariatric hypoglycemia following meal intake is caused by postprandial hyperinsulinemia, diagnosed by a mixed meal challenge test (MMCT) demonstrating hypoglycemia (glucose less than 55 mg/dL) accompanied by inappropriately elevated insulin (> 3.0 U/mL) and C-peptide (> 0.6 ng/mL)[9-10]. An important differential is the exclusion of a co-existing insulinoma[11-12] by cross sectional imaging or endoscopic ultrasonography, although these patients generally present with fasting hypoglycemia.

Postprandial hyperinsulinemic hypoglycemia following bariatric surgery was first described by Service *et al*[11] in a series of six patients who presented years after GBP surgery with neuroglycopenic symptoms and were found to have hyperinsulinemic hypoglycaemia. One patient was found to have an insulinoma, and the other five underwent pancreatectomy guided by intra-arterial calcium stimulation tests. Pathological examination showed islet cell hypertrophy and hyperplasia suggestive of nesidioblastosis and it was initially proposed that bypass surgery had resulted in beta cell hyperfunctioning and hyperinsulinemia. However other studies contest this finding[13], and other mechanisms proposed include an enhanced incretin effect[14], abnormal counter-regulatory hormone responses[15], altered enterohepatic circulation of bile acids[16] and changes in the microbiome[17]. The rapid transit of food from the stomach to the intestinal L cells is believed to result in an excessive release of incretins such as gastric inhibitory peptide and glucagon-like peptide 1 (GLP-1) in particular, with greater levels being

observed in symptomatic patients after meals[14].

The management of PBH is complex as its mechanisms remain incompletely understood. The majority of cases exhibiting mild symptoms respond to dietary modification, and medical nutritional therapy (MNT) is the cornerstone of management. The frequent intake of smaller meals comprising carbohydrates with a low glycaemic index helps prevent hypoglycaemia[18], with the intake of meals low in protein and/or high in sugars known to trigger these episodes[19]. Various pharmacological agents have been used with some success for patients who fail MNT, by blunting the inappropriately elevated insulin secretion and ensuing hypoglycemia. These include the alpha-glucosidase inhibitor acarbose, calcium channel antagonists like nifedipine or verapamil, the beta-cell adenosine triphosphate-sensitive potassium channel agonist diazoxide (inhibits insulin secretion by hyperpolarisation) and somatostatin analogues like octreotide[20]. Refractory patients may require a gastrostomy tube placement or a restrictive procedure, with some undergoing partial or total reversal of the bypass [9,20]. Over the years, GLP-1 has become an increasingly attractive target. A recent phase 2 randomised placebo-controlled crossover study (PREVENT) employing the GLP-1 receptor antagonist avexitide [exendin (9-39)] for 28 days showed a significant decrease in the occurrence of hypoglycemia in response to a MMCT requiring rescue as well as on continuous glucose monitoring, with an improved glycemic profile[21]. In another study[22], 12 participants with PBH were randomised to receive either glucagon or a placebo from an artificial pancreas system during meals as guided by a predictive algorithm using continuous glucose monitoring. The patients who received glucagon did not require rescue glucose or develop severe hypoglycemia (< 55 mg/dL), unlike those who received the vehicle, and thus mitigating severe hypoglycemia in PBH.

Elucidation of the other proposed mechanisms may guide the development of other safe and effective therapies for PBH.

FOOTNOTES

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