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EDITORIAL

Acarbose is again on the stage

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Abstract

Acarbose is an agent that has been used to treat type 2 diabetes for about 30 years; it prevents postprandial hyperglycemia by inhibiting carbohydrate digestion in the small intestine. Since incretin-based treatments have been preferred over the last 10 to 15 years, the use of acarbose is not as common in treating type 2 diabetes as before. Some studies have shown that acarbose also produces a weight-loss effect by increasing glucagon-like peptide 1 (GLP-1). The positive effect of acarbose on GLP-1, and increasing evidence that it provides cardiovascular protection, suggests that acarbose may again be considered among the first-choice antidiabetic agents, as it was in the 1990s.

Key Words: Acarbose; Cardiovascular protection; Glucagon-like peptide 1; Obesity; Waist-to-height ratio

Core Tip: The prevention of obesity and reducing cardiovascular risks, together with blood glucose control in patients with type 2 diabetes, are the main components of the treatment's goals. New studies show that acarbose can provide the expected benefits of an ideal antidiabetic drug by increasing both insulin sensitivity and glucagon-like peptide 1 levels.

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INTRODUCTION

Obesity is a key factor in the prevalence of type 2 diabetes mellitus (T2DM) worldwide. Therefore, in treating diabetes, researchers focus on the consequences of eliminating the negative effects of obesity, especially abdominal obesity, on reducing cardiovascular events and death. In a recently published study, Song *et al*[1] aimed to examine the effect of acarbose on abdominal obesity, and its determining factors in comparison with metformin[1]. They evaluated Metformin and AcaRbose in Chinese as the initial Hypoglycemic treatment (MARCH) study data[2] using a new anthropometric measure: Waist-to-height ratio (WHtR). The MARCH study is a randomized, open-labeled, noninferiority trial on Type 2 diabetes patients that was published in 2014[2]. It has been showen in this study that acarbose treatment is as effective and safe as metformin at the 24th and 48th weeks. A group of 343 patients who were newly diagnosed with T2DM were treated with acarbose, and 333 other patients were treated with metformin. The new report by Song *et al*[1] clarified that WHtR had significantly decreased in both groups in the 24th week after treatment, with women showing a more pronounced decrease. Between the beginning of the study and the 24th week of the treatment, the change in the waist-to-height ratio (ΔWHtR) was divided into two sets with large differences in one group and small differences in the other, thus, these data were subject to post-hoc analysis. In the acarbose group, women and those with a lower area under the glucagon-like peptide 1 (GLP-1) curve (AUCGLP-1) had a greater ΔWHtR. Among those using metformin, weight loss was greater in women as well as those with a high baseline AUCGLP-1. In conclusion, Song *et al*[1] found a relationship between high WHtR in the treatment of acarbose with gender, GLP-1 level, fasting glucose, and lipid profile. In addition, Song *et al*[1] emphasized the importance of WHtR for the measurement of abdominal obesity. They argued that, in both groups, a greater reduction in waist circumference in women was independent of the drug and was due to women's excessive desire and attempts to lose weight. The study observed that the circulating GLP-1 level increased over time in acarbose users. Previous studies reported that alpha glucosidase enzyme inhibition increased circulating GLP-1 levels by stimulating GLP-1 secretion and inhibiting dipeptidyl peptidase 4 (DPP-4) enzymes in healthy and T2DM patients[3-7]. Moreover, a recently published study showed this effect to be inhibited by exendin, a GLP-1 receptor antagonist[8]. This study found that acarbose is more effective for abdominal obesity, especially in those with low GLP-1 levels. The effect of lifestyle change on the results was not evaluated in the article, which is an important limiting factor.

The work of Song *et al*[1] throws up a question: "What role should acarbose play in the treatment of diabetes?" While acarbose continued to be part of diabetes guidelines and treatment algorithms, the appearance of new treatment agents in the last 10 to 15 years pushed acarbose to the background. In fact, there are large-scale studies that solidify the role of acarbose in treating impaired glucose tolerance (IGT) and T2DM. Over the past year, however, acarbose seems to have regained its importance. Prominent studies, such as the Study to Prevent Non-Insulin-Dependent Diabetes Mellitus (STOP-NIDDM) and the Acarbose Cardiovascular Evaluation (ACE) study, show that acarbose prevents the development of diabetes regardless of age, gender, and body mass index[9,10]. It has also been found that acarbose reduces cardiovascular events in patients with IGT and T2DM. In a recently published study, Zhang *et al*[11] found a 50% relative risk (RR) reduction in myocardial infarction and a 52% RR reduction in all-cause deaths after a 10-year follow-up with regard to acarbose therapy in patients with T2DM[11]. This effect is due to the reduction of oxidative stress caused by the lowering of postprandial two-hour blood sugar. Some studies have claimed that it is effective in quickly providing joint target controls. However, the fact that the study was conducted only in Chinese patients is an important limiting factor. An increasing number of studies focus on the mechanisms with which acarbose acts in diabetes treatment and how it provides additional benefits[8]. The possible effect mechanisms of acarbose on diabetic patients are shown in Table 1.

Acarbose inhibits carbohydrate digestion by competitively inhibiting the alpha glucosidase enzyme in the small intestine lumen. Consequently, it reduces glucose absorption, prevents postprandial hyperglycemia and hyperinsulinemia, and increases insulin sensitivity[12]. For this reason, it has been used in clinical practice since the 1990s, whether in monotherapy for mild cases of type 2 diabetes or as a combination agent with insulin and other antidiabetics in severe and advanced cases. Some studies have shown that acarbose has positive effects on intestinal flora[13]. In order to reduce gastrointestinal intolerance, a daily dose of 50 mg is offered just before meals, and a dose of 100 mg is offered three times a day after four to six weeks, when weekly titrations are reached. Acarbose can decrease hemoglobin A1c (HBA1c) by 0.5% to

Table 1 The possible mechanisms of effects of acarbose on diabetic patients

Type of effect	Net effect	Mechanism
Glucose absorption	Decrease	Competitively inhibits α-glucosidases absorption in small intestine
Insulin sensitivity	Increase	Lowers the postprandial blood glucose and insulin levels
DPP-4 activity	Decrease	Increases postprandial glucose in small intestine
Circulating GLP-1 level	Increase	Stimulates GLP-1 secretion in small intestine
Intestinal content	Increase	Positively effects microbiota *via* increasing content of oligosaccharides in the digestive tract

GLP-1: Glucagon-like peptide-1; DPP-4: Dipeptidyl peptidase 4.

1.5% and is especially effective on postprandial hyperglycemia[12].

The following are the advantages of acarbose: It is one of the rare agents that has been shown to prevent diabetes in the pre-diabetic period; the rate of hypoglycemia is low; its annual cost is lower than that of new antidiabetic drugs; it has weight-loss properties, or at least is weight neutral; it has a positive effect on the lipid profile by lowering the triglyceride level; and there is increasing evidence to show that it reduces the risk factors of cardiovascular disease. However, it shouldn't be forgotten that this hasn't yet been proven in Cardio Vascular Outcome Trials (CVOTs).

The disadvantages of acarbose are that it has to be used three times a day, and gastrointestinal side effects, such as gas, bloating, and diarrhea are relatively frequent.

CONCLUSION

In my opinion, we should remember that acarbose is an effective alternative to controlling postprandial hypoglycemia in countries that predominantly consume carbohydrates, like China or Turkey. The increasing evidence on its effects on GLP-1 and cardiovascular protection may lead to an extension of its use. It seems that acarbose, which has a high efficacy and is safe in terms of its side-effect profile, will be at the forefront of diabetes guidelines in the near future.

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REVIEW

Polycystic ovary syndrome and type 2 diabetes mellitus: A state-of-the-art review

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Abstract

Polycystic ovary syndrome (PCOS) often coexists with a wide spectrum of dysglycemic conditions, ranging from impaired glucose tolerance to type 2 diabetes mellitus (T2D), which occur to a greater extent compared to healthy body mass index-matched women. This concurrence of disorders is mainly attributed to common pathogenetic pathways linking the two entities, such as insulin resistance. However, due to methodological flaws in the available studies and the multifaceted nature of the syndrome, there has been substantial controversy as to the exact association between T2D and PCOS which has not yet been elucidated. The aim of this review is to present the best available evidence regarding the epidemiology of dysglycemia in PCOS, the unique pathophysiological mechanisms underlying the progression of dysglycemia, the most appropriate methods for assessing glycemic status and the risk factors for T2D development in this population, as well as T2D risk after transition to menopause. Proposals for application of a holistic approach to enable optimal management of T2D risk in PCOS are also provided. Specifically, adoption of a healthy lifestyle with adherence to improved dietary patterns, such the Mediterranean diet, avoidance of consumption of endocrine-disrupting foods and beverages, regular exercise, and the effect of certain medications, such as metformin and glucagon-like peptide 1 receptor agonists, are discussed. Furthermore, the maintenance of a healthy weight is highlighted as a key factor in achievement of a significant reduction of T2D risk in women with PCOS.

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Key Words: Polycystic ovary syndrome; Diabetes; Insulin resistance; Dysglycemia; Women

Core Tip: Polycystic ovary syndrome (PCOS) often coexists with a wide spectrum of dysglycemic conditions, ranging from impaired glucose tolerance to type 2 diabetes mellitus (T2D), which occur to a greater extent compared to healthy body mass index-matched women. This review provides the most current knowledge on the different aspects of T2D in women with PCOS, including epidemiology, common pathophysiologic mechanisms, and methodology employed for dysglycemia assessment, as well as to scrutinize the risk factors for T2D development and to suggests the optimal management of these women in the context of T2D risk reduction.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) constitutes the most common endocrine disorder in women of reproductive age, affecting 6%-15% of the of the global population[1]. PCOS is a multifaceted, ever-changing disease and a challenging disorder for the caring physician due to the continuous need for treatment modifications and adjustments based on the patient's fluctuating needs and preferences throughout the course of her lifetime. Apart from oligo- or amenorrhea and/or clinical or biochemical hyperandrogenism, impaired glucose homeostasis has also been observed in patients with PCOS[1,2]. In particular, evidence from large prospective cohorts has shown progression to either prediabetes or type 2 diabetes mellitus (T2D) over time[3,4]. The emergence of T2D in PCOS can be anticipated to some extent given that the two prerequisites for T2D development, insulin resistance (IR) and ß-cell dysfunction, are frequently present in women with PCOS. Indeed, IR, which is a key player in underlying PCOS pathophysiology, has been documented in the vast majority of women suffering from the syndrome in comparison to their healthy body mass index (BMI)-matched peers. An additive effect of obesity on the degree of IR reported in these women should also be taken into account[5]. Meanwhile, the prevalence of pancreatic ß-cell dysfunction is much higher in these patients compared to their regularly ovulating, non-hyperandrogenic peers[6].

Nevertheless, there is an ongoing debate as to whether PCOS itself constitutes a risk factor for T2D or whether T2D predominantly occurs in the context of obesity in affected patients[7-9]. A recent meta-analysis of genetic studies suggests that there is no inherent T2D risk in PCOS and that T2D instead occurs as a result of either increased adiposity or hyperandrogenemia[10]. On the other hand, PCOS constitutes a polygenic trait and elegant studies have shown that clusters of genes leading to metabolic disturbances are different from those associated with overt hyperandrogenic signs in PCOS women[11]. Therefore, a genetic component of dysglycemia among PCOS women should be considered.

The presence of altered glycemic status, although a universal finding, is challenging to the clinician for several reasons. One is that the reported incidence of dysglycemia, which includes impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and T2D, varies among studies. Furthermore, agreement over a definitive recommendation regarding the optimal method for assessment of glycemic status has not been reached to date.

The aim of this narrative review was to provide the most current knowledge on the different aspects of T2D in women with PCOS, including epidemiology, common pathophysiologic mechanisms, and methodology employed for dysglycemia assessment, as well as to scrutinize the risk factors for T2D development and to

suggest the optimal management of these women in the context of T2D risk reduction.

EPIDEMIOLOGY OF DYSGLYCEMIA IN PCOS

In general, the prevalence of dysglycemia is significantly higher in women with PCOS compared to their healthy BMI-matched peers. With regard to T2D, in normal women of reproductive age, the mean prevalence of T2D is 1%-3%[12], whereas in PCOS, its prevalence ranges from 1.5 to 12.4%, with a median value of 4.5%. This wide range partly depends on the age of the studied subjects, with the higher incidence (12.4%) recorded in a study evaluating mostly perimenopausal women with PCOS and a mean age of 46 years[13]. In the remaining studies, the mean age of the studied population ranged from 25 to 30 years. Another factor closely associated with T2D prevalence is ethnic variation, since a prevalence of 6.3% and 10.1% has been reported in two studies from Asia[14,15], reflecting the rising prevalence of T2D in Asia[16], a trend that has recently been corroborated in a large meta-analysis[17]. Finally, one more factor pertains to the criteria applied for PCOS diagnosis. For example, a higher degree of dysglycemia is anticipated in women diagnosed with the 1991 National Institutes of Health (NIH) criteria in comparison with the mild phenotype D of those diagnosed with the 2003 Rotterdam criteria, this due to the lower degree of IR observed in the latter group[18]. On the other hand, this logical assumption was not confirmed by a recent study evaluating more than 2000 women, which showed that T2D prevalence was similar among patients with different PCOS phenotypes[19].

Conflicting data exist regarding the prevalence of intermediate hyperglycemia, namely, IGT and IFG. The prevalence of IGT in PCOS ranges from 4%-35.4%, with an average of 16.6%; in contrast, the corresponding prevalence in the healthy peers of women with PCOS ranges from 4%-8%[12]. The reasons for this very high heterogeneity have not been fully elucidated; however, ethnic susceptibility, the various criteria applied for PCOS diagnosis, as well as age and BMI distribution in the different studied groups could partly explain this diversity. Likewise, IFG prevalence as reported in the literature ranges from 2%-21%, the average being 10.8%, higher than that of the non-PCOS population, in which it is approximately 5.9% (range 4%-8.7%)[20]. In addition to the reasons provided above, the diagnostic criteria employed to diagnose IFG also play an important role, with IFG cut-offs differing significantly between the American Diabetes Association (ADA) and the World Health Organization (WHO) clinical practice guidelines. Therefore, it is not surprising that the prevalence of IFG was higher in studies using the stricter ADA criteria[21] than in those using the corresponding WHO cut-off values[22]. The prevalence of dysglycemia as reported in different studies according to the country where the study was performed, the subjects' age and BMI, and the diagnostic criteria to confirm the diagnosis of either PCOS or glycemia are presented in Table 1.

PATHOPHYSIOLOGY LINKING PCOS WITH INCREASED RISK OF T2D

PCOS pathophysiology is characterized by a combination of androgen excess and ovulatory dysfunction. Although numerous studies have endeavored to identify the underlying pathogenetic mechanisms, this particular 'Holy Grail' of endocrinology has not as yet been uncovered. Irrespective of the theoretical perspective, it is widely accepted that the unusually variable phenotype in affected patients is produced by the combined effects of two separate, yet deeply intertwined, mechanisms, which are androgen over-activity (elevated androgen concentrations or hyperandrogenism) and IR[23].

IR represents a state of disrupted insulin binding to its receptor or ineffective activation of the latter by insulin, thereby forcing the pancreatic β-cells to release large amounts of insulin into the circulation in order to maintain euglycemia[24]. Such a state of chronic pancreatic stress leads to impaired glucose homeostasis, initially manifesting as IFG or IGT; however, once large numbers of islet β-cells have succumbed to stress, it leads to T2D. IR and glucose homeostasis abnormalities have been described in up to 70% of women with PCOS[25]. As early as 1980, eight obese subjects with PCOS were found to have higher serum glucose and insulin concentrations, in both a fasting state and after stimulation by an oral glucose load, compared with six obese unaffected women, despite the latter being statistically significantly more obese[26]. Even though obesity is a key risk factor for IR and T2D development in the general population, women with PCOS have higher insulin concentrations in

Table 1 Incidence of dysglycemia in women with polycystic ovary syndrome

Ref.	Sample size	Country	PCOS criteria	T2D criteria	Age (yr)	BMI (kg/m²)	IFG (%)	IGT (%)	T2D (%)
Rajkhowa *et al*[142], 1996	90	UK	NIH	WHO	26 (15-39)	31.6 (18-48)	?	9	2
Legro *et al*[61], 1999	254	USA	NIH	WHO	14-44	32 ± 3	?	31	7.5
Ehrmann *et al*[62], 1999	122	USA	NIH	ADA	25 ± 0.7 (13-40)	30-43	9	35	10
Gambineri *et al*[3], 2004	121	Italy	Rotterdam	WHO	14-37	20-38	?	15.7	2.5
Legro *et al*[143], 2005	71	USA	NIH	ADA	30 ± 6	29 ± 6.4	?	25	10
Chen *et al*[144], 2006	102	China	Rotterdam	WHO	24.2 ± 6	21.7 ± 4	?	20.5	1.9
Mohlig *et al*[64], 2006	264	Germany	NIH	WHO	28 ± 0.4	30 ± 0.4	?	14.3	1.5
Vrbikova *et al*[145], 2007	244	Czech Republic	Rotterdam	ADA	27 ± 7.5	27 ± 6.9	12.3	9.4	1.6
Gagnon *et al*[146], 2007	105	Canada	NIH	ADA	28.3 (14-47)	35.5 (19-54)	?	23	5
Dabadghao *et al*[63], 2007	372	Australia	Rotterdam	ADA	30 ± 5 (15-62)	35 ± 8	3	15.6	4
Espinos-Gomez *et al*[147], 2008	102	Spain	NIH	WHO	26 ± 6	30.2 ± 8	?	10.7	7.7
Cheung *et al*[148], 2008	295	China	Rotterdam	ADA	30 ± 6	25 ± 5.9	9.2	10.5	7.5
Bhattacharya *et al*[149], 2009	264	India	Rotterdam	WHO	24 ± 4	27 ± 4.5	?	14.4	
Seneviratne *et al*[15], 2009	168	Sri Lanka	Rotterdam	WHO	29 ± 4 (20–40)	25.92 (16-39)	?	23.2	10.1
Lee *et al*[50], 2009	194	Korea	Rotterdam	ADA	27 ± 5	24 ± 4	17		1
Wei *et al*[51], 2009	356	China	Rotterdam	WHO	32 ± 4 (19-44)	22 ± 4.2	?	7.6	**3,1**
Zhao *et al*[150], 2010	818	China	Rotterdam	ADA	25 ± 5	?	8.5	35.4	**4**
Stovall *et al*[151], 2011	78	USA	NIH	ADA	26 ± 6.4	29 ± 6 (18-43)	2	14	**?**
Celik *et al*[66], 2013	252	Turkey	Rotterdam	ADA	24 ± 5	26 ± 5.7	?	14.3	**2**
Veltman-Verhulst *et al*[21], 2013	226	Netherlands	Rotterdam	ADA	29.6 ± 4	27 ± 6.7	21	4	**3.5**
Lerchbaum *et al*[152], 2014	714	Austria	Rotterdam	ADA	27 (23-32)	24.2 (21-30)	12.8		1.5
Vrbikova *et al*[145], 2014	330	Czech Republic	Rotterdam	ADA	27.8 ± 7	27.6 ± 6	12	8.8	**3**
Amato *et al*[22], 2015	241	Italy	Rotterdam	WHO	24 ± 6 (14-43)	30 ± 6 (18-50)	11.6	5.4	**1.7**
Ganie *et al*[14], 2015	2014	India	Rotterdam	ADA	23 ± 5.4	25 ± 4.4	14.5	5.9	**6.3**
Gracelyn *et al*[153], 2015	200	India	Rotterdam	ADA	16-40	?	?	14.5	**1.5**
Li *et al*[154], 2016	2436	China	Rotterdam	ADA	27	21.56	13.5	19.8	**3.9**
Ollila *et al*[127], 2017	265	Finland	Rotterdam	WHO	46	28.6 ± 6	?	?	**12.4**
Pelanis *et al*[13], 2017	876	Sweden	Rotterdam	ADA	29 (25-34)	28 (23-33)	11	12	**3**
Zhang *et al*[19], 2018	378	China	Rotterdam	IDF	27 ± 4.4	30 ± 4.3	31.5		8.7
Ortiz-Flores *et al*[155], 2019	400	Spain	Rotterdam	WHO	26 (14-49)	28.6 (22-34)	14	14.5	2.5

NIH: National Institutes of Health; T2D: Type 2 diabetes mellitus; PCOS: Polycystic ovary syndrome; ADA: American Diabetes Association; WHO: World Health Organization.

response to an oral glucose load as compared to unaffected subjects, even in the absence of obesity[27,28]. The only clinical sign of IR is acanthosis nigricans, which correlates well with IR in either obese or lean affected individuals[29].

Of course, women with PCOS are equally exposed to the well-established association of obesity and higher degree of IR as those without PCOS. Indeed, a study comparing 198 obese and 201 non-obese women with PCOS (obesity definition: BMI > 27 Kg/m²) found that obesity was associated with lower insulin sensitivity when a

variety of oral glucose tolerance test (OGTT)-derived indices was used[30]. In contrast to the latter findings, a study employing the impractical gold standard method to assess IR, namely, the hyperinsulinemic euglycemic clamp, it found more pronounced insulin secretion in lean women with PCOS compared to controls, though without significant differences in insulin sensitivity, while it confirmed the presence of higher IR compared to controls only in obese women with PCOS[31].

In addition, women with PCOS present with enhanced luteinizing hormone pulsatility, producing increased secretion of ovarian and adrenal androgens, which, along with IR, are key features of the syndrome. A meta-analysis of cross-sectional studies including 4795 women from the general population found higher testosterone concentrations in patients with T2D compared to controls[32]. In addition bioavailable testosterone correlated significantly with IR, with higher concentrations predicting the development of T2D[33]. Similar results were obtained from a systematic review and meta-analysis pooling data from the Rotterdam Study and other previously published studies, which found that subjects at the highest tertile of free androgen index had a 42% higher risk of developing T2D, in a complex multivariate analysis controlling, among others, for age, BMI, glucose, and insulin concentrations[34].

Given that PCOS is characterized by markedly higher androgen concentrations compared to those in the unaffected population, an association between the syndrome and T2D constitutes a rational hypothesis. In fact, such correlations were originally reported almost 40 years ago[35]. Ever since, multiple studies have confirmed this relationship in both lean and obese women with PCOS. A significant positive association between testosterone concentrations and IR has been described in lean women with PCOS using OGTT-derived indices[3,36] and measures of glucose disposition in hyperinsulinemic euglycemic clamps[37]. Even though PCOS is mainly characterized by hyperandrogenemia of ovarian origin, a subgroup of patients exhibits adrenal androgen hypersecretion, with the most important being dehydroepiandrosterone sulphate. In the latter subgroup, hyperandrogenemia does not seem to correlate with IR indices or metabolic abnormalities in most[38-42], but not all, studies[43,44]. It is a riddle that remains as yet unresolved, especially taking into consideration that such an association does seem to exist in other conditions of adrenal hyperandrogenism, such as premature adrenarche/pubarche[45].

Despite the similar pathophysiology of PCOS and glucose homeostasis abnormalities, the differences between lean and obese women with PCOS are remarkable. This was shown by a recent study in which lean women with PCOS failed to improve their whole body insulin action, measured using a hyperinsulinemic euglycemic clamp after 14 wk of controlled and supervised exercise training, in contrast to controls[46]. Therefore, it came as no surprise when a recent meta-analysis of 13 studies including almost 279000 subjects identified a markedly elevated risk for T2D among women with PCOS compared to unaffected women [5.9% *vs* 2.0%; relative risk (RR): 3.00, 95%CI: 2.56-3.51; I^2 = 83%][47]. Similar results were found *via* a meta-analysis recently presented by our group: in this systematic review, 23 studies were lumped together, incorporating data from 319870 participants who comprised 60337 patients with PCOS and 8847 cases with T2D (RR: 3.45, 95%CI: 2.95-4.05). In our study, the effect of BMI on the risk of T2D was assessed, pooling data from three studies, which identified a pronounced effect of obesity. In particular, the RR for developing T2DM in overweight/obese and non-obese women with PCOS, as compared to their non-PCOS counterparts, was 5.75 (95%CI: 1.20-27.42) and 3.34 (95%CI: 0.03-400.52), respectively. Moreover, the RR for developing T2D in overweight/obese compared to lean women with PCOS was 3.96 (95%CI: 1.22-12.83)[48].

Meanwhile, the role of aging in T2D development should certainly not be underestimated. This has been demonstrated in, inter alia, a subgroup analysis of 345 Dutch women with PCOS, who were part of a large cohort study evaluating aging in women with PCOS (APOS study)[49], where the interaction of age and BMI was the most significant variable in predicting T2D in logistic regression analysis. Moreover, data assembled from several studies have also pointed to a positive association of age and BMI with T2D or intermediate hyperglycemia among women with PCOS[19,50,51]. On the other hand, a cross-sectional study conducted by our group found that aging might exert a protective effect in women with PCOS with regard to IR. In particular, obese women with PCOS demonstrated the same degree of IR through the years, although this was not the case for their lean peers in whom a gradual improvement was observed with aging (Figure 1)[52]. Furthermore, a large cross-sectional study (n = 763 normal-weight women with PCOS, according to the Rotterdam criteria; 376 controls) exhibited a parallel decrease of homeostasis insulin resistance assessment (HOMA-IR) index with free androgen index, suggesting a potential mechanism regulating this process (Figure 2)[53]. Specifically, the gradual reduction of androgen

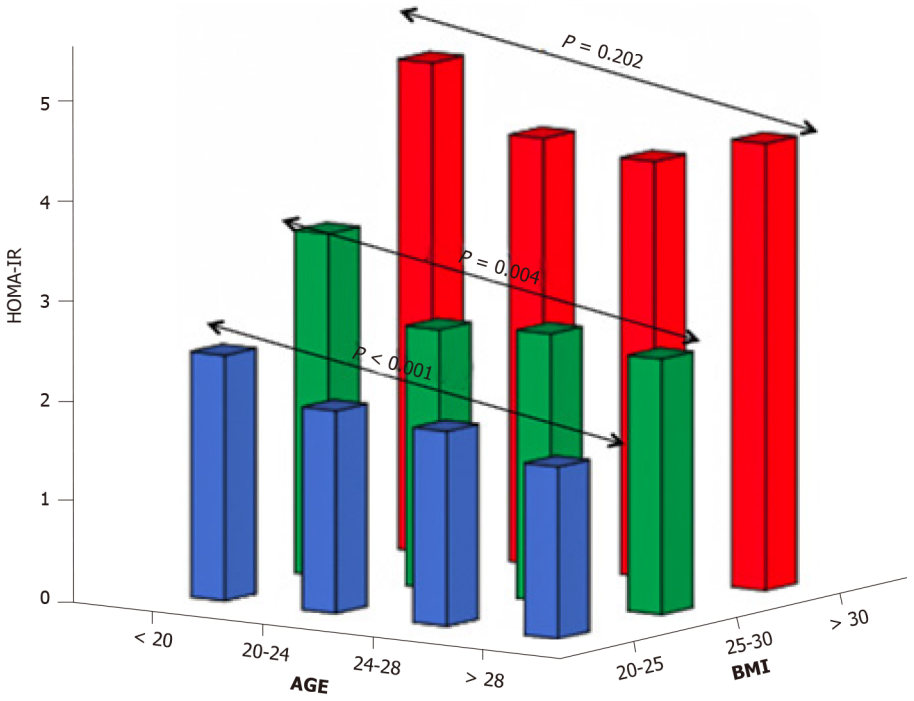

Figure 1 The gradual improvement of insulin resistance over the years in normal weight (blue bars) and overweight (green bars) women with polycystic ovary syndrome, but not in their obese counterparts (red bars). Adapted from[52]. HOMA-IR: Homeostatic model assessment for insulin resistance; AGE: Advanced glycation end-product; BMI: Body mass index.

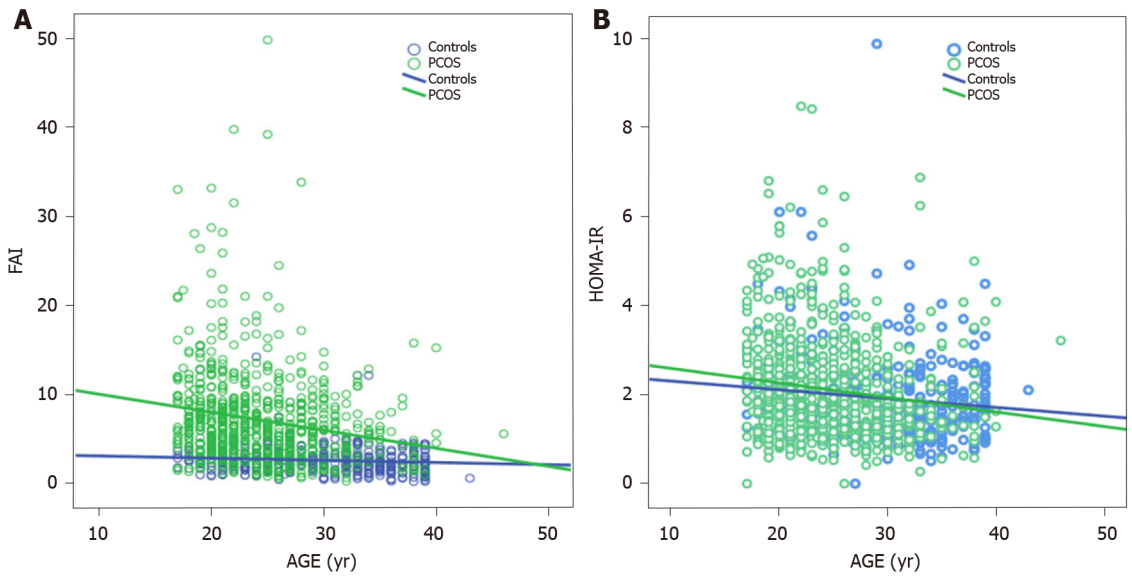

Figure 2 The gradual decrease of homeostatic model assessment for insulin resistance and free androgen index in normal weight women with polycystic ovary syndrome, compared with controls. Adapted from[53]. A: Free androgen index; B: Homeostatic model assessment for insulin resistance. PCOS: Polycystic ovary syndrome; FAI: Free androgen index; HOMA-IR: Homeostatic model assessment for insulin resistance.

production observed in women with PCOS after their third decade of life partly explains the absence of deterioration of IR through the years which is common in the general population.

ASSESSMENT OF DYSGLYCEMIA IN PCOS

According to the Wilson and Jungner criteria, screening for a disease is essential when that condition constitutes an important health problem, an accepted treatment for patients with recognized disease exists, and facilities for diagnosis and treatment are

available. Furthermore, there should be an identifiable latent or early symptomatic stage, and the natural history of the condition, including development from latent to clinical disease, must be adequately understood[54]. Furthermore, there should be an agreement upon policy as to whom to treat, taking into consideration the patients' resources. The cost of case finding (including diagnosis) should be economically balanced in relation to possible expenditures for medical care as a whole, while case finding should be a continuing process and not a "one-off" strategy. Finally, there should be an effective screening test or examination and that test should be acceptable to the population[54]. It is obvious that PCOS covers all the prerequisites described above and, therefore, in all consensus statements by several experts, screening for T2D is recommended in women with PCOS (Table 2).

However, whether glycemic status should be evaluated in every woman suffering from PCOS or in certain subgroups, as well as which is the best method for this assessment, are to date unanswered questions. With regard to which patients should be screened, there are at present two points of view. One, supported by the Endocrine Society, the Androgen Excess and Polycystic Ovary Syndrome Society, as well as by the guidelines on PCOS diagnosis and management developed in Australia, suggests universal screening in all women with PCOS[55-57]. On the other hand, a number of experts recommend screening in women with at least one risk factor, such as age >40 years, family history of either T2D or gestational diabetes mellitus, and/or obesity[58-60]. However, the latter recommendations have not been supported by solid data, since studies arguing either in favor of or against them have been published[4,61]. For example, the family history of T2D criterion has strong supportive data in studies from the USA and Australia[62,63], but not in studies originating from Italy and Germany[9,64]. Accordingly, these criteria, although reasonable, appear ultimately to be arbitrary and would not reflect the different nature of T2D development in PCOS compared to that in the unaffected population. In a similar manner, most of the studies in favor of these recommendations did not evaluate in detail the impact of age, obesity, and hyperandrogenemia on the development of dysglycemia and, thus, seem to increase controversy over this matter[4,65].

There is disagreement among experts as to whether fasting plasma glucose (FPG), OGTT, or glycated hemoglobin (HbA1c) is the best laboratory method to assess glycemic status in a patient with PCOS, despite robust data strongly pointing to OGTT as being the most accurate[66,67]. The main arguments against OGTT use are that the modality is more complex, expensive, and time-consuming than the other two screening methods. Moreover, it is characterized by high variability and its results are dependent on height[68]. However, OGTT is considered the gold standard for T2D diagnosis because it constitutes a standardized test that is easily performed and is the only method able to detect IGT, of utmost importance for women with PCOS[69]. Indeed, given that the risk of T2D development in women with IGT is considerably higher than in those with normal glucose tolerance or those with IFG[9], this at-risk population can greatly benefit from early lifestyle modification and/or pharmacological intervention[70].

In addition, the ADA and WHO are in agreement regarding the glucose concentration cut-off for the diagnosis of IGT, which is not the case for either FPG or HbA1c values. Furthermore, in several studies it has been shown that a single measurement of FPG could misclassify a substantial number of patients with either IGT or T2D, ranging from 20%-40%, as having normal glucose homeostasis[21,64,71]. This figure is certainly not negligible given that women with PCOS are at risk for T2D, even from their early reproductive years, compared to their healthy peers. Other benefits of an OGTT are that it can be applied in patients with iron deficiency, a condition commonly encountered in women of reproductive age, while the parallel measurement of insulin concentrations after a glycemic load provides the clinician with an accurate estimate of the degree of existing IR[72].

After the ADA's recommendation for a single HbA1c measurement as an accurate index for T2D diagnosis, its use has been advocated by several research groups and international guidelines (Table 2). HbA1c cannot, however, be used for the diagnosis of dysglycemia in women suffering from PCOS for a number of reasons. First, in this group of patients, periods of oligomenorrhea are followed by periods of heavy bleeding or sychnomenorrhea and this menstrual pattern could result in major changes in hematocrit and/or ferritin levels. Since HbA1c is dependent on these parameters, iron depletion and loading might lead to significant variations in HbA1c concentrations over time, independently of the patient's glycemic status[73]. Moreover, the specificity of HbA1c in the diagnosis of dysglycemia has been questioned in overweight and obese subjects[74], who constitute the largest group in the PCOS population. Additionally, the cut-off point for HbA1c is mainly based on the

Table 2 Guidelines regarding oral glucose tolerance test upon diagnosis of Polycystic ovary syndrome

Ref.	OGTT recommended upon diagnosis in all women with PCOS	Follow-up with OGTT
Joint AACE/ACE and AE-PCOS society[56]	Yes	(1) Yearly in women with IGT; and (2) Every 1–2 years based on BMI (not specified) and family history of T2D
Australian NHMRC[57]	Recommended if one or more criteria exist: (1) BMI > 25 kg/m^2 or in Asians > 23 kg/m^2; (2) History of IFG, IGT, GDM; (3) Family history of T2D; (4) Arterial hypertension; and (5) High-risk ethnicity	Every 1-3 years, based on presence of other diabetesrisk factors
Endocrine Society[55]	Yes	Every 3–5 years (Sooner if additional risk factors for T2D develop)
Royal College of Obstetricians andGynecology[59]	Recommended if one or more criteria exist: (1) BMI ≥ 25 kg/m^2; (2) Age ≥ 40 years; (3) Previous GDM; and (4) Family history of T2D	Yearly in women with IGT or IFG
AE-PCOS Society[58]	Recommended if one or more criteria exist: (1) BMI ≥ 30 kg/m^2; (2) Age ≥ 40 years; (3) Previous GDM; and (4) Family history of T2D	Every 2 years in women with risk factors (Sooner if additional risk factors for T2D develop)
ESHRE and ASRM[60]	Recommended if BMI ≥ 27 kg/m^2	Not specified

OGTT: Oral glucose tolerance test; PCOS: Polycystic ovary syndrome; T2D: type 2 diabetes mellitus; BMI: Body mass index; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance.

established association between HbA1c and microvascular disease in patients with established T2D. However, women with PCOS are younger and healthier overall when initially diagnosed with the syndrome, while dysglycemia does not always lead to T2D in this population compared to those evaluated in the original study of HbA1C validation[75], further calling HbA1c application into question.

Besides this, HbA1c is a costly procedure, harmonization of HbA1c assays around the globe has not yet been carried out effectively, significant variation across ethnicities has been described, and international standardization is not as yet complete [76]. The diagnostic performance of HbA1c as a marker of glucose intolerance is further compromised by the discordance between the diagnostic criteria for prediabetes proposed by the WHO [42 mmol/mol (6.0%)] and those by the ADA [39 mmol/mol (5.7%)], producing much confusion. Furthermore, available studies evaluating the ability of HbA1c to detect IGT and diabetes in PCOS have found that the test has low sensitivity when compared with OGTT for the assessment of glucose tolerance[66,67]. Finally, the diagnostic accuracy of HbA1c in detecting T2D has recently been questioned, with some investigators arguing strongly in favor of OGTT for this procedure[77].

There is, moreover, much discordance among experts regarding the frequency of glycemic status assessment, ranging from yearly to on a five-year basis depending on the coexistence of additional factors. All the latter recommendations are illustrated in Table 2. However, from a pathophysiological point of view, PCOS women with IFG constitute a different subgroup from those with IGT. In fact, data derived from a healthy population have shown that isolated IFG is usually observed in subjects with predominantly hepatic IR and normal muscle insulin sensitivity, whereas individuals with isolated IGT have normal to slightly reduced hepatic insulin sensitivity and moderate to severe muscle IR[78]. Accordingly, those with IFG may represent the general population of women prone to T2D development, whereas subjects with IGT may compose that group of patients in whom dysglycemia occurs as a consequence of hyperandrogenemia. Today, in fact, copious documentation of the detrimental effects of androgens on muscle insulin sensitivity in lean women with PCOS is underway[1].

The conversion rate from normal glucose homeostasis to IGT or from IGT to T2D in PCOS has been estimated to range from 2.5 to 3.6% annually over a period of 3–8 years [79-81]. These conversion rates are lower than those observed in individuals with IGT in the general population, who seem to develop T2D at rates of approximately 7% annually[82]. This discrepancy may be related to the fact that the underlying mechanisms of T2D development in PCOS are different from those found in healthy individuals. In fact, in non-PCOS women, the degree of insulin sensitivity progressively deteriorates, thus leading almost inevitably to T2D. In contrast, in women with PCOS, a diversity of IR values has been observed over time, appearing to improve in lean subjects and worsen only when obesity is present[52,53]. Non-linear progression to T2D is therefore quite frequent in subjects suffering from PCOS, and

this strongly correlates with their degree of adiposity. This notion is further supported by Celik *et al*[83] who showed that obese women with PCOS had a 4-fold greater risk of converting from normal glucose tolerance to IGT. The latter finding was additionally corroborated by Rubin *et al*[4] who demonstrated that lean women with PCOS had an equal risk of progressing to T2D to that of controls.

RISK FACTORS FOR THE DEVELOPMENT OF T2D IN PCOS

Several parameters which could possibly mediate the risk of developing T2D in women with PCOS have recently been evaluated. With regard to PCOS phenotypes, the presence of the most severe form of PCOS, consisting of the conglomeration of chronic anovulation and elevated androgen concentrations (former NIH criteria), was found to be one of the strongest independent predictors of glucose concentrations after a 75-g OGTT in 254 women with PCOS following adjustment for several confounders, including age, waist-to-hip ratio (WHR), and BMI[61]. However, this finding has not since been replicated[13].

Based on the well-known developmental origins of health and disease, an inverse association between birth weight and T2D risk seems highly likely. Indeed, low birth weight has been associated with PCOS diagnosis later in life, with a birth weight < 2.5 kg conferring a 76% higher likelihood of developing PCOS[84]. In a similar manner, age of menarche has been related to dysglycemia in PCOS. Indeed, PCOS women with IGT were observed to have significantly earlier menarche age (11.9 ± 1.6 years) compared to obese women with PCOS and normal glucose tolerance (12.4 ± 1.7 years) in a cross-sectional study of 121 Italian PCOS women. However, the number of subjects with T2D was too small for any correlation to be established[3]. Of note, a single-center cohort study demonstrated that a higher number of births decreased the risk of T2D in women with PCOS, corroborating a potential prophylactic effect of parity in T2D[4]. In the same context, the impact of lactation on T2D development may be hypothesized. In fact, obesity in women with PCOS is a risk factor for impaired lactogenesis and increases the risk for reduced breastfeeding initiation and duration [85]. Furthermore, since lactation is crucial for women's post-gestational metabolic health[86], the presence of abnormal lactational function might enhance the risk for T2D in this population, even though substantial data to support this hypothesis are to date lacking. Finally, a positive link has been proposed between family history of T2D and T2D risk in PCOS; this hypothesized association is based on a defect in the first phase of insulin secretion in PCOS women with a first degree relative suffering from T2D, in contrast to BMI-matched PCOS patients without such a history[87].

Indisputably, the impact of environmental factors on T2D development is major. Endocrine disruptors constitute an emerging environmental threat, and a role for endocrine disrupting chemicals in exacerbating PCOS pathology has been proposed for over 20 years, with bisphenol A (BPA) being significantly associated with measures of IR and BMI in women with the syndrome[88]. Moreover, a positive feedback loop between BPA and hyperandrogenemia has been shown in PCOS and, therefore, BPA exposure has been particularly incriminated in PCOS pathophysiology[89]. Furthermore, the role of advanced glycation end-products (AGEs) in the pathophysiology of T2D development in PCOS is another issue that has gained ever more attention over time. AGEs are products of non-enzymatic glycation and oxidation (glycoxidation) of proteins and lipids in both hyperglycemic and euglycemic states. Thermally processed foods, mostly lipid- and protein-rich foods typical of Western diets, are responsible for the exceedingly high intake of exogenous AGEs, these remaining in the body and being incorporated covalently in different tissues. Dietary AGEs have been associated with subclinical inflammation and endothelial dysfunction both in patients with T2D and in unaffected individuals[90]. Of interest, concentrations of AGEs are elevated in women with PCOS compared to their healthy counterparts independently of the degree of obesity and IR[91]. They are positively associated with androgens and anti-Müllerian hormone levels and are localized in human polycystic ovaries. Based on all the above, a potential role of AGEs in the PCOS machinery has been suggested[90]. In women with PCOS, consumption of a diet high in AGEs was followed by a deterioration of IR and hyperandrogenemia, whereas elimination of AGEs was followed by a significant amelioration of these key parameters, even without a change in BMI[92]. On the other hand, the link of endocrine disruptors with human pathophysiology should be interpreted with caution, since their actions are exerted in an non-monotonic pattern[93].

Three of the most common addictions have been incriminated in PCOS pathophysiology and, possibly, in the development of T2D, namely, smoking, caffeine, and alcohol. Regarding tobacco use, a cross-sectional study of 309 women with PCOS identified a significant positive correlation between lipid concentrations and years of smoking, while IR was significantly higher in smokers with PCOS compared to non-smokers, despite the absence of a significant association between HOMA-IR and pack-years among the participants[94]. In addition, HOMA-IR and fasting insulin concentrations were higher in smokers with PCOS compared with their non-smoker counterparts in a German study of 346 women with PCOS, although the latter analysis was not adjusted for BMI or age[95]. Regarding caffeine, higher intake has been associated with worse reproductive outcomes in women with PCOS, but no study to date has found any association with glucose homeostasis[96]. Finally, a positive association between alcohol consumption and PCOS has been documented[97].

Another risk factor contributing to impaired glucose homeostasis may be vitamin D deficiency. Regarding PCOS, lower 25-hydroxy-vitamin D [25(OH)D] concentrations have been reported in PCOS patients compared to those in controls, with vitamin D deficiency [25OHD < 20 ng/mL] being associated with higher fasting glucose and insulin concentrations, as well as IR, assessed by OGTT[98]. A meta-analysis combining data from 11 placebo-controlled randomized trials evaluating the effects of vitamin D supplementation on glucose homeostasis in 601 patients with PCOS (89% of Asian descent) found that daily supplementation with small doses of vitamin D was able to significantly lower HOMA-IR index (daily supplementation effect -0.30; P = 0.0018, low-dose supplementation effect −0.31; P = 0.0016)[99]. However, both studies failed to report data regarding the relationship between vitamin D deficiency and T2D.

Taking a more holistic approach, other emerging factors in T2D development are sleep quality and mood disorders. It is well-known that women with PCOS, even after controlling for obesity, tend to have a higher prevalence of sleep disturbances, such as reduced sleep efficiency, amount of time spent in rapid eye movement (REM) sleep, as well as non-REM sleep, and difficulty in falling asleep and maintaining sleep[100]. Moreover, the prevalence of obstructive sleep apnea is higher in women with PCOS compared to non-PCOS [odds ratio (OR) 3.83; 95%CI: 1.43-10.24][101] and is associated with higher fasting insulin levels, HOMA-IR index, HbA1c, and glucose area under the curve[102]. On the other hand, the latter findings warrant caution, since a high likelihood for selection bias is considered plausible[101]. Depression and mood disorders have been associated with IR, obesity, and T2D in multiple studies[103]. In addition, depression and mood disorders have been commonly diagnosed in women with PCOS[104,105]. Despite the theoretical possibility of an association between these two conditions, no study has evaluated such an association to date.

In recent years, the role of the gut microbiome in metabolic abnormalities, including PCOS, has been explored[106]. It was thus inevitable that an effort would be made to improve some of the features of the syndrome by intervening in the microbial population with probiotics and synbiotics. The latter intervention was shown to confer beneficial effects on body weight, fasting plasma glucose and insulin, reproductive hormones, and hirsutism, while longer duration of treatment also seemed to be more efficacious[107].

A major factor that may eliminate or reduce T2D risk in PCOS is prescription of appropriate drugs. Oral contraceptives have been the mainstay of treatment for irregular menses, hirsutism, and acne in women with PCOS with exceptional success rates. Some studies have, however, suggested an increased risk for T2DM with this strategy[4], albeit a meta-analysis of published trials identified only a minor increase in fasting insulin concentrations[108]. By contrast, given the significance of IR in the pathophysiology of the syndrome, it comes as no surprise that metformin has been the most commonly used medication to prevent or treat the metabolic abnormalities in women with PCOS[109]. Yet, despite the high expectations, metformin combined with lifestyle changes appeared to produce only a small reduction in BMI (-0.73 kg/m^2). This was, namely, a decrease in subcutaneous adipose tissue volume and an improvement in menstrual cyclicity compared to lifestyle interventions alone, these as seen in a meta-analysis of published randomized controlled trials[109]. It is, however, of note that most studies were small in sample size and the risk of bias was deemed high by the authors. Patients with concurrent diabetes were excluded in most studies, not allowing for safe conclusions to be drawn in this regard. On the other hand, metformin significantly reduced the risk for gestational diabetes (pooled OR 0.20, 95%CI: 0.12-0.1, P < 0.001), early pregnancy loss (pooled OR 0.28, 95%CI: 0.10-0.75, P = 0.01), and preterm delivery (pooled OR 0.33, 95%CI: 0.18-0.60, P = 0.0003), with significant heterogeneity between studies[110]. These outcomes did not differ between patients treated prior to pregnancy and those treated throughout the duration of their

pregnancy[111].

In addition to metformin, PPAR-γ agonists, such as rosiglitazone (not used currently due to heart failure risk) and pioglitazone, which are potent insulin sensitizers, have been studied in women with PCOS, exhibiting significant improvements in fasting blood glucose and glucose tolerance[25]. In 2017, a meta-analysis of 11 randomized controlled trials comparing the effects of metformin and pioglitazone in 643 subjects with PCOS identified a number of promising effects on reproductive and esthetic outcomes and, as expected, metformin seemed to ameliorate BMI more effectively than pioglitazone[112]. Other T2D medications have been studied in women with PCOS recently, including the SGLT-2 inhibitor empagliflozin, which produced significantly more weight loss compared to metformin in a small study of non-diabetic women[113]. Exenatide[114,115] and liraglutide[116] appeared to improve several glucose homeostasis parameters in non-diabetic women with PCOS more efficaciously than metformin, while their addition to metformin seemed to provide incremental benefits in that regard[116,117]. Finally, orlistat has been studied extensively in obese women with PCOS and was found equally efficacious to metformin in producing weight loss and metabolic improvements[118].

Even though supplement use has increased greatly in the past few years, supplementation with minerals, trace elements, and other supplements is, in general, still controversial. In the case of chromium supplementation in PCOS, two meta-analyses of published trials found that BMI, fasting insulin, free testosterone[119] HOMA-IR, and HOMA-β[120] seemed to improve. In addition, supplementation with omega-3 fatty acids at doses of 900-4000 mg daily appeared to improve HOMA-IR index in a meta-analysis of nine small randomized controlled trials, but no data were available on risk for T2D[121]. Myoinositol, an amino acid with potentially beneficial effects in women with PCOS, has been studied with regard to glucose metabolism, and a small positive impact on fasting insulin and HOMA-IR was found in a 2017 meta-analysis of controlled trials, without any effect on glucose concentrations or BMI[122]. The factors described above and their relationship to development of dysglycemia in PCOS are illustrated in Figure 3.

T2D IN POSTMENOPAUSAL WOMEN WITH PCOS

Based on the aforementioned data, a diagnosis of PCOS during the reproductive age places a woman at increased risk for T2D in later, post-reproductive life[123]—a risk which is further augmented if the issue of dysregulated glucose metabolism during transition to menopause is taken into account. The risk is even greater if the woman enters menopause before the age of 45[124]. A recent meta-analysis of 23 cohort studies[47] assessed the long-term cardiovascular disease (CVD) risk in women with PCOS, including T2D risk. Women with a history of PCOS demonstrated a 3-fold increased risk of T2D (RR 3.00; 95%CI: 2.56-3.51) compared to non-PCOS women. However, the studies included were quite heterogeneous in terms of the participants' age. Very few of them included postmenopausal women, either as a single or a mixed population[8,125,126]. Another shortcoming was the heterogeneity in PCOS diagnosis among studies.

A recent prospective cohort study evaluated the risk for development of T2D in 27 PCOS women (defined by the NIH criteria) after 24 years of follow-up (mean age at baseline: 29.5 ± 5.3years; mean age at the end of follow-up: 52.4 ± 5.4 years) in comparison with age-matched non-PCOS controls (n = 94). The incidence of T2D in the former group was 19% compared to 1% in the latter ($P < 0.01$)[8]. Interestingly, the development of T2D was independent of lifestyle factors. Although all PCOS women with T2D were obese and had a higher BMI and WHR than non-PCOS individuals, the increases in BMI and WHR per year were comparable between groups during follow-up. However, an older prospective study including 35 postmenopausal women with PCOS (mean age 70.4 ± 5 years, range 61-79), diagnosed with the Rotterdam criteria, did not find any difference in T2D incidence compared to age-matched controls after 21 years of follow-up[125]. Moreover, a retrospective cohort study published in 2000, which included 319 PCOS women (mean age 56.7 years, range 38-98; 81% postmenopausal; PCOS diagnosis based on medical records) and 1060 age-matched controls, showed a 3-fold increased risk of T2D in the PCOS group (OR 2.8; 95%CI: 1.5-5.5) after a mean follow-up time of 31 years (range 15-47). However, this risk was not significant after adjustment for BMI (OR 2.2; 95%CI: 0.9-5.2)[126].

In general, whether the increased T2D risk exists in both obese and non-obese PCOS has not as yet been fully elucidated. A recent meta-analysis by Zhu *et al*[10] that

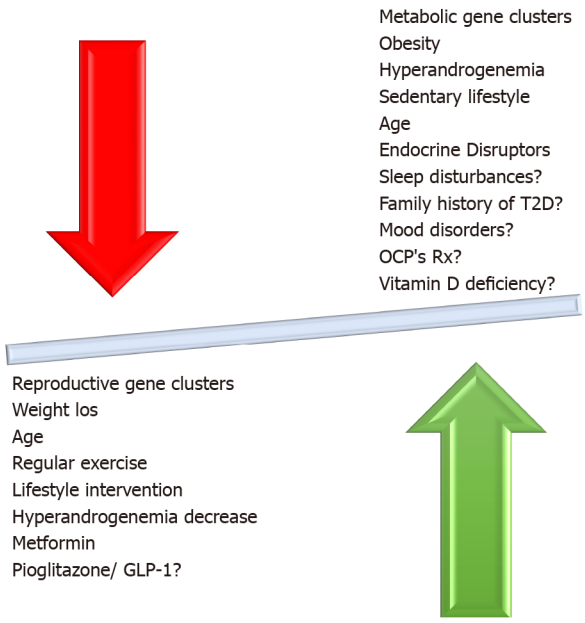

Figure 3 The interaction of positive (green arrow) and negative (red arrow) factors affecting dysglycemia in women with polycystic ovary syndrome. T2D: Type 2 diabetes mellitus; GLP-1: Glucagon-like peptide 1.

assessed the risk of T2D in non-obese PCOS compared to non-obese control women showed an increased risk in this PCOS subpopulation, although to a lesser extent compared to obese PCOS (five studies; OR 1.47; 95%CI: 1.11–1.93). The authors additionally reported an increased prevalence of IR, IGT, and atherogenic dyslipidemia in non-obese PCOS compared to non-obese non-PCOS women. It must be underlined that the PCOS populations in the included studies were all premenopausal [10]. Of note, a prospective cohort study of this meta-analysis assessed the incidence of T2D at the age of 46 years in a cohort of 279 women with both oligoamenorrhea and hirsutism at the age 31 years, who had been defined as "PCOS". This cohort was compared with 1577 women, without oligoamenorrhea and hirsutism, who served as controls. Women with PCOS and BMI > 25 kg/m^2 demonstrated a 2.5-fold increased risk of T2D compared to non-PCOS (OR 2.45; 95%CI: 1.28–4.67). It is notable that no such risk was identified in PCOS women of normal weight[127]. A very recent case-control study (1136 PCOS patients, aged 15 to 44 years, and 5675 controls) showed an increased risk of T2D in PCOS independently of BMI (adjusted OR in the entire cohort 2.36, 95%CI: 1.79–3.08; OR in non-obese PCOS 2.33, 95%CI: 1.71–3.18; OR in obese PCOS 2.85, 95%CI: 1.59–5.11)[128].

Aside from BMI, other factors also play an important role in the incidence of T2D in postmenopausal women with a history of PCOS. The increased ovarian androgen production and IGT observed in premenopausal women with PCOS cases seem to persist after menopause transition. On the other hand, IR and hyperinsulinemia may improve in women with PCOS during their post-reproductive years, although data are still inconsistent as to this hypothesis[129]. Furthermore, the severity of IR is also dependent on the PCOS phenotype, since hyperandrogenemia is related to a more severe metabolic dysfunction[65].

Therefore, although transition to menopause is associated with dysregulation of glucose metabolism[130], current evidence is at present too weak to support the existence of increased T2D risk in postmenopausal women with a history of PCOS compared to those without. There are currently too many confounding factors and variables, such as PCOS definition, sample size, and the precise effect of BMI and aging, to accurately determine the actual impact, if any, of a PCOS diagnosis on T2DM risk after transition to menopause.

MANAGEMENT OF T2DM RISK IN PCOS

Lifestyle intervention, including diet modification and regular exercise, still remain the mainstay of treatment in reducing T2D risk in women with PCOS, especially those who are obese or overweight. According to a recent meta-analysis of 19 randomized

controlled trials (RCTs), systematic dietary intervention is superior to advice, usual diets, or no treatment with regard to HOMA-IR [mean difference (MD) -0.78, 95%CI: -0.92 to -0.65], fasting plasma insulin (FPI) (MD -4.24 mIU/L, 95%CI: -5.37 to -3.10), FPG (MD -0.11 mmol/L, 95%CI: -0.17 to -0.04 mmol/L), as well as BMI (MD -1.01 kg/m^2, 95%CI: -1.38 to -0.64) and waist circumference (WC) (MD -3.25 cm, 95%CI: -5.29 to -1.22)[131]. Subgroup analysis showed that the Dietary Approaches to Stop Hypertension (DASH) diet is more effective in HOMA-IR and FPG reduction than a low-carbohydrate diet (LCD), but with comparable efficacy regarding FPI concentrations. With respect to BMI and body weight, a calorie-restricted diet is more beneficial than either DASH or LCD. All dietary patterns seem to have comparable efficacy regarding WC[131]. Moreover, data from RCTs in PCOS women have shown that LCD is quite effective in reducing BMI [standardized MD (SMD) -1.04, 95%CI: -1.38 to -0.70) and HOMA-IR (SMD -0.66, 95%CI: -1.01 to -0.30) compared to a regular diet according to a recent meta-analysis[132]. In addition, a low glycemic index diet could also be the first-line approach in PCOS patients, since it effectively reduces HOMA-IR (-0.78, 95%CI: -1.20 to -0.37), WC (-2.81 cm, 95%CI: -4.40 to -1.23), and total testosterone concentrations (-0.21 nmol/L, 95%CI: -0.32 to -0.09) compared to a high glycemic index diet[133]. Although the evidence in PCOS populations is limited, the Mediterranean diet (MedDiet) compared to the typical Western diet is also effective in reducing HOMA-IR (MD -0.42, 95%CI: −0.70 to −0.15), FPG (MD -2.98 mg/dL, 95%CI: -4.54 to -1.42), and FPI (-0.94, 95%CI: -1.72 to -0.16) compared to a usual diet. The MedDiet is also associated with a lower tendency to develop T2DM (RR 0.81; 95%CI: 0.61-1.02) and a reduction in CVD events related to metabolic syndrome[133].

The beneficial effects of structured exercise programs in metabolic syndrome, obesity, T2D, and CVD prevention and treatment are well-known[134]. Interventions consisting of lifestyle modifications in women with PCOS produce substantial improvements in glucose homeostasis and reproductive outcomes as well. These benefits are equally significant as those achieved by metformin[135]. A negative energy balance of approximately 30%, aiming to achieve an energy deficit of 500-750 kcal per day, is able to produce significant amounts of weight loss in women with PCOS. The addition of any amount of exercise, whether aerobic or anaerobic, confers additional beneficial effects on glucose homeostasis[25]. High-intensity interval training (achieving 90%-95% of the individual's maximum heart rate) three times a week for ≥ 10 wk is effective in reducing HOMA-IR (MD -0.57, 95%CI: -0.98 to -0.16) and BMI (MD -1.90, 95%CI: -3.37 to -0.42) in women with PCOS, according to a recent meta-analysis[136]. In cases with morbid obesity, such as those with BMI > 40 kg/m^2 or BMI > 35 kg/m^2 with metabolic comorbidities, bariatric surgery should be considered[1]. Indeed, bariatric surgery can reduce the risk of T2D by 91% (RR 0.09, 95%CI: 0.03-0.32). The mean reduction in BMI is -14.51 kg/m^2 (95%CI: -17.88 to -11.14). It also ameliorates menstrual disturbances and hirsutism in PCOS patients [RR 0.23 (95%CI: 0.13-0.43) and 0.47 (95%CI: 0.28-0.79), respectively][137].

Metformin is the most commonly used insulin sensitizer in women with PCOS, especially in those who are obese or overweight. According to the aforementioned meta-analysis, diet is superior to metformin with regard to weight loss, but comparably efficacious in improving glucose homeostasis (HOMA-IR, FPG, and FPI) [131]. In a recent meta-analysis of 12 RCTs, metformin was superior to placebo in reducing BMI [weighted MD (WMD) -1.25, 95%CI: -1.60 to -0.91] and WC (WMD -1.41, 95%CI: -2.46 to -0.37). There were no differences between groups with regard to HOMA-IR, FPG, and FPI[138].

For women who are intolerant to metformin, thiazolidinediones (TZDs) constitute another class of insulin sensitizers that have been evaluated in women with PCOS. Rosiglitazone and pioglitazone, the two commonly used TZDs, are effective in improving IR and IGT, as well as mensural cyclicity, in PCOS patients. However, weight gain, increase in transaminase levels, and potential teratogenic effects limit their use in these patients[1]. Compared to metformin, pioglitazone is superior with respect to menstrual cycle improvement and ovulation but inferior regarding hirsutism score. Both agents are equally effective in reducing HOMA-IR, FPG, and FPI, as mentioned above[112].

Glucagon-like peptide 1 (GLP-1) receptor agonists (GLP-1-RAs) have also been tested in women with PCOS. A recent meta-analysis of eight RCTs (four with exenatide 10 μg twice a day; four with liraglutide 1.2 mg/d), compared their efficacy with that of metformin in women with PCOS. The study showed that GLP-1-RAs were more effective in improving HOMA-IR (SMD -0.40, 95%CI: -0.74 to -0.06) and reducing BMI (SMD -1.02, 95%CI: -1.85 to -0.19) and WC (SMD -0.45, 95%CI: -0.89 to -0.00). No difference between GLP-1-RAs and metformin in FPG and FPI was observed either between exenatide and liraglutide[139].

Finally, in cases of post-menopausal women younger than 60 years and/or within 10 years since their last menstrual period who present with severe vasomotor symptomatology, especially those with early menopause (< 45 years of age), menopausal hormone therapy (MHT) may be of benefit, since it reduces T2D by up to 30%[140]. MHT exerts a beneficial effect on glucose homeostasis in women both with and without T2D. In cases with T2D and low CVD risk, oral estrogens may be considered [141]. In obese women with T2D or with moderate CVD risk, transdermal 17β-estradiol is the preferred treatment, along with a progestogen with minimal effects on glucose metabolism, such as progesterone, dydrogesterone, or transdermal norethisterone. However, this favorable effect on glucose homeostasis is dissipated after MHT discontinuation. In any case, MHT is not recommended for the sole purpose of T2D prevention or treatment[140,141].

It is thus clear that weight loss, preferably with LCD and a low glycemic index diet low in AGES, combined with vigorous exercise should be the first-line lifestyle intervention in overweight or obese PCOS patients due to their well-documented beneficial effects on glucose metabolism, although longitudinal data on T2D risk are thus far lacking. The MedDiet may be even more beneficial than a low glycemic index diet in CVD risk reduction, although the current evidence in PCOS patients is weak. Metformin may also be considered in cases of impaired glucose metabolism and oligo/amenorrhea. The choice of either BS, pioglitazone, or GLP-1-RA should be individualized and benefits should be weighed against costs.

CONCLUSION

The association of PCOS with increased T2D risk is relatively robust and thus should not be neglected in any woman with the syndrome. Despite the current heterogeneity of the data, the ever-changing nature of this disorder, and the uncertainty regarding the exact mechanisms regulating progression of dysglycemia in PCOS, there are several general principles that the clinician should implement in everyday practice. First, diagnosis and follow-up of dysglycemia should preferably be based on OGTT and not on FPG or HbA1c values. Second, the non-linear development of T2D in PCOS in non-obese women highlights the importance of maintaining an optimal weight in all women suffering from the syndrome. Third, menopausal women with a history of PCOS should be regularly evaluated since they may be at higher T2D risk, especially if they are obese. Fourth, a well-balanced diet coupled with regular exercise constitutes the most appropriate approach in every patient with PCOS. Metformin administration might ameliorate the biochemical and hormonal profile in PCOS and may be considered in patients in whom prior measures have failed to improve metabolic and ovulatory dysfunction. The use of pioglitazone, GLP-1-Ras, and/or MHT may be of value in selected cases.

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MINIREVIEWS

Management of diabetic foot ulcers and the challenging points: An endocrine view

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Abstract

Diabetic foot ulcers (DFU) are one of the most challenging complications of diabetes. Up to one-third of patients with diabetes mellitus (DM) may suffer from DFUs during their life. DFU is one of the leading causes of morbidity in patients with DM. The treatment period is challenging, and the recurrence rate of DFUs is high. Hence, establishing prevention strategies is the most important point to be emphasized. A multidisciplinary approach is necessary in the prevention and treatment of DFUs. Patients at risk should be identified, and prevention measures should be taken based on the risk category. Once a DFU is formed, the appropriate classification and evidence-based treatment interventions should be executed. Glycemic control, diagnosis and treatment of vascular disease, local wound care, diagnosis, and treatment of infection should be addressed along with the proper evaluation and management of general health status.

Key Words: Diabetic foot; Diabetic foot ulcer; Amputation; Diabetic foot infection

Core Tip: Diabetes mellitus is a chronic disorder with dramatic complications. Nearly one-third of patients with diabetes may suffer from foot ulcers during their life. A potentially preventable event usually has dramatic results. The prevention and management of diabetic foot ulcers (DFUs) necessitate a multidisciplinary approach. The most important approach is the prevention of the formation of DFU. Prevention measures should be implemented in a timely manner, and adequate treatment interventions should be executed immediately once it is formed.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder that has become a global health problem in the last decades[1]. DM has several complications that affect not only life expectancy but also the quality of life[2,3]. Diabetic foot ulcers (DFU) are one of the most challenging complications of DM. Up to one-third of diabetic patients may suffer from DFUs during their life[4,5]. The global prevalence of DFUs is reported at 6.3%, with DFUs being more common in men than women and in type 2 DM than type 1 DM[6]. The recurrence rate of DFUs is also high. The value reaches 40% within 1 year and 65% within 3 years[4]. Hence, studies should focus on establishing prevention strategies against DFU[4,5].

PATHOPHYSIOLOGY AND PREDISPOSING FACTORS

Peripheral artery disease (PAD) and diabetic neuropathy (DNP) are well-known chronic complications of diabetes[7]. Along with immune dysfunction, PAD and DNP are the main pathophysiological factors that predispose to DFUs[8]. DFUs are associated with DM duration, the presence of DNP, and PAD[9]. DNP is present in 80% of patients with DFUs, and it facilitates ulcer formation by causing decreased pain and pressure sensation. DNP also promotes the formation of anatomic deformities, such as prominent plantar metatarsal heads, hammertoes, Charcot foot, *etc.*[4,10]. Patients with diabetes should be assessed for DNP periodically after the diagnosis of type 2 DM and after the fifth year of type 1 DM. Pain, burning, and numbness should be questioned. Small fibers (by pinprick test and temperature sensation), large fibers (by vibration perception and 10 g monofilament test), and protective sensation (by 10 g monofilament test) should be tested. The tests predict the risk of complications besides screening the dysfunction[7,11,12].

Nearly half of the patients with DFUs have PAD, which is significantly associated with the increased risk of adverse limb events[13]. Vascular symptoms, including reduction in effort capacity, leg fatigue, and claudication, should be assessed. All peripheral pulses should be palpated together with an assessment of extremity appearance and warmth to evaluate perfusion[8,13]. Patients should also undergo the ankle-brachial index (ABI) testing as a part of the examination. The normal value of ABI is between 0.9 and 1.3, which is higher than 1.0[13,14]. A high ABI may be measured falsely in the presence of vascular calcifications[13]. Toe-brachial index (TBI) measurement is also recommended, especially in combination with ABI and arterial Doppler study. The diagnosis of PAD is unlikely in the presence of triphasic Doppler waveforms when the TBI is ≥ 0.75, and the ABI is between 0.9-1.3[13]. In addition, disrupted blood flow may be present at the microvascular level despite the intact or well-treated macrovascular component[15]. Dysfunctional signs of blood flow at the microvascular level can be detected by laser Doppler flowmetry[16]. Furthermore, DM causes immunological dysfunctions at the cellular level, leading to poor healing response and susceptibility to infections[8,17].

CLINICAL SIGNIFICANCE

DFUs are a serious healthcare problem globally. A potentially preventable event, such as a minor trauma, usually has dramatic results. DM remains the primary cause of nontraumatic lower-limb loss worldwide[18-20]. DFUs pose a serious financial burden worldwide, and nearly one-third of expenses for DM is estimated to be for DFUs[21-23]. The presence of DFU is associated with the increased risk of mortality in DM, and this association is stronger than the presence of any macrovascular disease alone[3,24]. The five-year survival rate in patients presenting with DFUs is poorer than that associated with the most common cancers[21]. Therefore, the best approach in the management of DFUs is the implementation of preventive measures based on the risk

class[7,10,25].

IDENTIFICATION AND FOLLOW-UP OF PATIENTS AT RISK

DNP, PAD, foot deformity, and medical history of DFU are the most important risk factors for new DFU formation. These factors are the shadows of the coming event, which is DFU if the preventive measures are not applied in time[4,10,26]. Poor glycemic control, chronic kidney disease (especially dialysis), and smoking are also among the risk factors[7,8]. Diabetic patients should be categorized based on the risk of developing DFU. Thus, the risk factors for DFUs must be screened at least annually [7,12]. The risk classification system developed by the International Working Group on the Diabetic Foot (IWGDF) is useful in daily clinical practice (Table 1)[13].

A diabetic patient with very low risk (IWGDF group 0) must be examined annually for DNP and PAD. The patients who have a higher risk (IWGDF group 1-3) should be examined more frequently (Table 1), and preventive measures should be executed (Table 2)[7,13]. Patients who have moderate-to-high risk should wear therapeutic shoes to reduce plantar pressure and the risk of ulceration. Pre-ulcerative lesions, abundant callus, stinging toenails, and fungal infections (tinea pedis, onychomycosis, *etc*.) should be treated properly. Surgical interventions should be performed to fix deformities, if necessary[7,10,13]. The patient's feet with DNP should be inspected every visit, and the patients at risk should be encouraged and educated about self-care and preventive measures[7].

CURRENT EVIDENCE FOR PREVENTION

Several randomized clinical trials (RCT) evaluated the primary prevention strategies of DFUs, but none of them were high-quality research[27]. Conducting RCT to determine the primary prevention strategies and evaluate their efficacy is a considerable challenge, given that numerous patients and a long follow-up period will be required [21]. On the other hand, conducting RCT on the prevention of ulcer recurrence is technically easier because the recurrence rate is high[4,21]. Suitable therapeutic footwear with appropriate pressure distribution prevents recurrence or worsening of plantar foot ulcers, with high-quality evidence[7].

DNP and PAD are the major predisposing factors of DFU development[28,29]. Thus, the neurosensory and vascular systems of the extremities must be protected to prevent or delay the development of DFUs. The onset and progression of diabetic microvascular complications (retinopathy, nephropathy, and neuropathy) can be delayed by intensive glycemic control. This finding has been shown in type 1 DM, but the current evidence in type 2 DM is weak[30-32]. However, no specific therapeutic agent or approach other than glycemic control can modify the progression of microvascular complications[7,10].

PAD is one of the macrovascular complications of diabetes. The benefit of intensive glycemic control on macrovascular complications in diabetics has not been shown in RCTs, but several epidemiological analyses reported a correlation between an increased rate of cardiovascular disease (CVD) and chronic hyperglycemia[33-35]. The benefit of intensive therapy could not be shown in three large RCTs comparing intensive and conventional therapies in terms of cardiovascular benefits in patients with longstanding DM[36,37]. Unlike these studies, in a research investigating the effect of glycemic control on complications in newly diagnosed DM, the benefit of intensive glycemic control on CVD was shown after a 10-year follow-up on the post-interventional period[38]. The management of other CVD risk factors is particularly important in the prevention or delay of PAD and other macrovascular complications in patients with DM. Smoking cessation, effective treatment of hyperlipidemia and hypertension, weight loss, appropriate nutrition, and exercise habits are important points that should be emphasized in every patient with DM. Exercise should be considered with caution if the patient is in the risk group for DFU. Patients in the low- or moderate-risk group should be advised exercises that increase the motion of foot and ankle, relieve pressure, and decrease neuropathic symptoms. Patients in the risk group should avoid long walks, exercises that increase the pressure on the soles of feet, activities with a risk of trauma, and wearing inappropriate shoes[13].

Table 1 International Working Group on the Diabetic Foot risk classification system

Group	Definition	Ulcer risk	Screening
0	No LOPS and no PAD	Very low	Once a year
1	LOPS or PAD	Low	Once every 6-12 mo
2	LOPS + PAD or LOPS + FD or PAD + FD	Moderate	Once every 3-6 mo
3	LOPS or PAD with one or more of the following: (1) History of a foot ulcer; (2) A lower extremity amputation (major or minor); and (3) ESRD	High	Once every 1-3 mo

LOPS: Loss of protective sensation; PAD: Peripheral artery disease; FD: Foot deformity; ESRD: End-stage renal disease.

Table 2 Preventive measures for diabetic foot ulcers

	Preventive measures
1	Avoid smoking
2	Avoid walking barefoot/in socks without shoes/in thin-soled slippers
3	Avoid hot ground and hot sand
4	Inspect both feet and inside the shoes daily
5	Wash the feet daily (carefully dry especially between the toes)
6	Test water temperature before bath
7	Lubricate dry skin and avoid chemicals
8	Cut the toenails straight
9	Do not remove callus
10	Wear snug shoes (customize if feet have deformity)
11	Change the socks daily

CURRENT TECHNOLOGICAL OPPORTUNITIES FOR MONITORING

The recurrence rate of DFUs is also extremely high in patients who are under follow-up in specialized centers. Thus, systems that facilitate recognition of the early signs of DFU formation must be developed. Patients can refer to health care providers early, and preventive and/or therapeutic appropriate strategies can be executed on time[42]. Risky conditions for DFU formation, such as early signs of inflammation and pressure-induced plantar tissue stress by current technological opportunities, can be screened and followed-up[29]. The available technological devices had been invented for this purpose; these devices include instruments for daily monitoring plantar temperature, socks that enable temperature monitoring continuously, socks that monitor plantar pressure, smart insoles to screen sustained plantar pressure, alarm systems that warn patients to wear offloading devices, activity monitoring devices, *etc*.[29].

POINTS TO BE CONSIDERED IN DFU MANAGEMENT

DFU is the major cause of nontraumatic lower extremity amputations (LEA), worldwide[20,39]. Once DFU occurs, the management strategies should be implemented without delay. Numerous studies emphasized the importance of a multidisciplinary team approach in the management of these patients[20,40,41]. The multidisciplinary team should focus on four major points; glycemic control, diagnosis and treatment of vascular disease, evaluation and local management of wound, diagnosis, and treatment of infection[41].

Glycemic control

The importance and role of adequate glycemic control for delaying or preventing chronic complications of DM are discussed above. Although RCTs have shown an association between intensive glycemic control before DFU formation and the low risk of LEAs, to our knowledge, the role of glycemia in the management of active DFU has never been studied in RCTs[42,43]. Considering the known negative effect of hyperglycemia on wound healing and immune defense, hyperglycemia may be associated with negative consequences in patients with DFUs[8,17,44]. Several meta-analyses of observational studies addressed this point[43,45,46]. Margolis *et al*[46] published a meta-analysis of five observational studies including DFUs. Glycemic control was not associated with wound healing according to this study. The other two meta-analyses reported that the high fasting plasma glucose and Hba1c levels were associated with a high rate of amputations[43,45].

In addition to the effect of hyperglycemia on the wound healing process, hyperglycemia causes impaired immune functions and decreased response to infections[20,47]. American Diabetes Association (ADA) and American Association of Clinical Endocrinologists (AACE) recommend targeting glucose levels between 140-180 mg/dL without causing hypoglycemia in the majority of inpatients[48]. These levels should be aimed at patients with DFUs treated in inpatient setting.

An intercurrent illness (trauma, infection, surgery, *etc.*) causes impaired glycemic control in diabetics and necessitates adjustment of the therapy. Here, DM patients are predisposed to severe hyperglycemia, diabetic ketoacidosis, and nonketotic hyperosmolar state. Patients treated with noninsulin antidiabetics require insulin. ADA and AACE recommend insulin regimens for critically ill and noncritically ill hospitalized patients[48].

Several oral antidiabetics have other properties besides the glucose-lowering effect. For instance, canagliflozin, a sodium-glucose cotransporter-2 (SGLT-2) inhibitor, is associated with an approximately two-fold increased risk of LEA (primarily at the level of toe or metatarsal) in patients with type-2 DM and established CVD (or at risk for CVD) *vs* placebo[49]. On the other hand, in RCTs of empagliflozin and dapagliflozin, the risk of amputation was similar between the treatment and placebo arms [50]. An increased risk of LEAs was reported with canagliflozin, empagliflozin, and dapagliflozin (for toe amputations) in a pharmacovigilance study. This study relied on several LEA cases[51]. Recent meta-analyses found no associations between SGLT-2 inhibitors and increased LEA risk; however, Chang *et al*[53] compared the use of SGLT-2 inhibitors with other oral antidiabetics and reported that SGLT-2 inhibitors may contribute to the increased risk of LEA[49,52]. A study examining systematic reviews, which evaluated the adverse effects of SGLT-2 inhibitors, summarized the scarcity of high-quality systematic reviews on this topic[54]. To our opinion, SGLT-2 inhibitors may increase the risk of LEA in patients with DFU as a group effect. Conflicting data are available regarding this traffic; thus, exercising cautiousness is reasonable.

Vascular disease

The prevalence of PAD among DFU patients reaches 50%. The presence of PAD is significantly related to adverse limb events. All patients with DFU should be examined clinically for PAD. Doppler sonographic study should be performed with a combination ABI and/or TBI test. No single modality has been defined as optimal. Vascular imaging (and revascularization if PAD is present) should always be considered when the ulcer remains unhealed in 4-6 wk despite the appropriate treatment and normal condition (ABI and TBI)[7,13]. The threshold for performing vascular studies should be very low in DFU patients, especially for those who are unresponsive to treatment[55]. Based on the vascular structure and clinical conditions, surgical bypass or endovascular treatment can be applied as a revascularization therapy[15,55].

Local wound management

The first step in the treatment of DFUs is to classify the wound and assess the patient's medical condition. The depth and width of the ulcer, the presence of ischemia, and infection should be evaluated. Classification systems have been developed for DFUs (Table 3). Wound classification helps in the prediction of prognosis, along with determining the type and intensity of treatment[20,56,57]. All infected and nonvitalized tissues should be removed by surgical debridement, and the abscess should be drained, if present[58]. Other debridement methods, such as mechanical, enzymatic, and biological debridement, are available other than surgical procedures[20]. Surgical

Table 3 Classification systems of diabetic foot ulcers

Classifications system	The evaluated parameters
University of Texas System	Depth, infection, ischemia
Wagner	Depth, necrosis
PEDIS	Perfusion, extent, depth, infection, sensation
SINBAD	Site, ischemia, neuropathy bacterial infection, area, depth
Threatened limb classification: WIfI	Wound characteristics, ischemia, foot infection
IWGDF/IDSA system	Clinical manifestations, the severity of infection, PEDIS grade

IWGDF: International Working Group on the Diabetic Foot; IDSA: Infectious Disease Society of America.

debridement is the most effective and preferred method[20,58].

Post-debridement wound care is vital. Further tissue injury should be avoided. Proper wound coverage and dressing are necessary. Negative pressure therapy can be used if the wound is clean. Wound characteristics are determinative of the dressing procedure. Pressure reduction is another important point for wound healing. Several available methods of mechanical offloading (cast walkers, wedge shoes, bed rest, *etc.*) are also applied. Surgical pressure reduction may be needed occasionally[20,56,57].

Management of infection

DFUs are predisposed to infection. The exact diagnosis of infection should be performed correctly the first time to manage the infection in DFUs. The classical manifestations of inflammation (warmth, erythema, tenderness, and swelling), extent of infection, involvement of deep tissues and/or bones, and presence of an abscess and/or fistula tract should be evaluated. The clinician should be acquainted with the clinical findings of necrotizing infections. Systemic manifestations of infection (including findings of systemic inflammatory response syndrome and sepsis) and hemodynamic status should also be assessed carefully along with the wound characteristics[58,59]. The presence of severe infection, extensive gangrene, necrotizing infection, deep abscess, compartment syndrome, and/or limb-threatening ischemia needs immediate consultation with a surgeon[59].

Most diabetic foot infections are polymicrobial. A wound specimen must be obtained for culture if no clinical sign of infection is observed[56]. However, the specimens for culture should always be collected in the presence of infection (especially in moderate-to-severe infection) before antibiotic administration[56,59]. Specimens for culture can be collected by aspiration of the abscess, curettage from the ulcer (after debridement), or biopsy during the surgical procedure (from deep tissue or bone) but not by superficial swab[58,59].

Empiric antimicrobial therapy should be considered in the presence of infection, and the selection of antibiotic should be based on clinical findings and the severity of infection[56,58,59]. An antibiotic regimen that covers gram-positive organisms only is preferable in antibiotic-naive patients with mild infections. In the case of antibiotic treatment in the last several weeks of, severely ischemic limb, or moderate-to-severe infections, the coverage of antibiotic therapy should include commonly isolated gram-negative organisms and anaerobes (in certain conditions) besides gram-positive organisms[59]. The clinical course and culture results should drive antibiotic therapy during follow-up[56,59].

POTENTIAL ADJUNCTIVE THERAPIES

In addition to all these interventions, several adjunctive therapies may help the healing of DFUs [negative pressure wound therapy (vacuum-assisted closure), skin grafts and substitutes, hyperbaric oxygen therapy, shock wave therapy, growth factors, autologous combined leucocyte, platelet, fibrin, and placental derived products][56,60]. No high-quality evidence supports the recommendation of these interventions without concern, and none of these treatments is an alternative to the best standard therapy[60].

CONCLUSION

A DFU is a challenging complication of diabetes that has become a global health problem. The treatment process is troublesome for the patient and healthcare team, and the treatment results are often unsatisfactory, especially in advanced cases. Moreover, the recurrence rate is high despite the healing of ulcer. DFUs are one of the leading causes of morbidity in diabetic patients.

DFUs are potentially preventable. Hence, strict implementation of primary and secondary prevention strategies should be implemented. However, the scarcity of high-quality evidence especially in establishing preventive measures for primary prevention is a challenge.

The multidisciplinary team approach is the cornerstone of the management of DFU. All the team members should be experienced in their field. The evidence-based standard follow-up and treatment algorithms should be applied without delay once an ulcer develops.

Geographic heterogeneity in terms of access to adequate healthcare equipment and experienced healthcare team, poor adherence of the patients, late reference to health care providers, difficulties in achieving adequate perfusion of ulcer, the presence of DNP, the impossibility of restoring sensation, and high recurrence rates are the featured challenging points in the management of DFU.

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ORIGINAL ARTICLE

Basic Study

High doses of catecholamines activate glucose transport in human adipocytes independently from adrenoceptor stimulation or vanadium addition

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Author contributions: Carpéné C designed the studies, performed the cell experiments, reviewed the literature and wrote and revised the manuscript; Grolleau JL obtained human biological resource; Boulet N isolated the cells for *in vitro* studies; Morin N was involved in data mining, contributed to the literature review and revised the manuscript.

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Abstract

BACKGROUND

When combined with vanadium salts, catecholamines strongly activate glucose uptake in rat and mouse adipocytes.

AIM

To test whether catecholamines activate glucose transport in human adipocytes.

METHODS

The uptake of 2-deoxyglucose (2-DG) was measured in adipocytes isolated from pieces of abdominal subcutaneous tissue removed from women undergoing reconstructive surgery. Pharmacological approaches with amine oxidase inhibitors, adrenoreceptor agonists and antioxidants were performed to unravel the mechanisms of action of noradrenaline or adrenaline (also named epinephrine).

RESULTS

In human adipocytes, 45-min incubation with 100 μmol/L adrenaline or noradrenaline activated 2-DG uptake up to more than one-third of the maximal response to insulin. This stimulation was not reproduced with millimolar doses of dopamine or serotonin and was not enhanced by addition of vanadate to the

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incubation medium. Among various natural amines and adrenergic agonists tested, no other molecule was more efficient than adrenaline and noradrenaline in stimulating 2-DG uptake. The effect of the catecholamines was not impaired by pargyline and semicarbazide, contrarily to that of benzylamine or methylamine, which are recognized substrates of semicarbazide-sensitive amine oxidase. Hydrogen peroxide at 1 mmol/L activated hexose uptake but not pyrocatechol or benzoquinone, and only the former was potentiated by vanadate. Catalase and the phosphoinositide 3-kinase inhibitor wortmannin inhibited adrenaline-induced activation of 2-DG uptake.

CONCLUSION
High doses of catecholamines exert insulin-like actions on glucose transport in human adipocytes. At submillimolar doses, vanadium did not enhance this catecholamine activation of glucose transport. Consequently, this dismantles our previous suggestion to combine the metal ion with catecholamines to improve the benefit/risk ratio of vanadium-based antidiabetic approaches.

Key Words: Human adipocytes; Amine oxidases; Insulin; Diabetes; Vanadium; Obesity

Core Tip: Our recent results indicated that the combination of catecholamines plus vanadium strongly stimulates glucose transport in rat adipocytes. We therefore proposed that catecholamine/vanadate salts could lead to the development of novel derivatives exhibiting potent insulin-like properties. Here, we found that adrenaline and noradrenaline stimulated glucose transport in human adipocytes but in a manner that was not dependent on and not enhanced by the presence of vanadate. Consequently, our previously proposed usefulness of the synergism of catecholamines/vanadium does not work in human fat cells. This might hamper the improvement of vanadium-based antidiabetic approaches, limited so far by toxicological issues.

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INTRODUCTION

In a recent report, we demonstrated that catecholamines such as noradrenaline and adrenaline (also named norepinephrine and epinephrine) are capable of activating glucose transport in rodent adipocytes, essentially in the presence of vanadium[1]. These observations, providing the basis for novel research of vanadium/amine complexes exhibiting antidiabetic properties of the metal ions with less toxicological issues, needed further verification. Particularly, the demonstration of relevance to humans was lacking for this alleged insulin-like effect of high doses of catecholamines.

To extrapolate to humans our recent description of a stimulatory effect of catecholamines plus vanadium on glucose transport in rodent fat cells[1], we have reproduced our previous explorations in human adipocytes. Although cultured preadipocytes undergoing *in vitro* adipogenesis and immortalized cell lines have been successfully used to document the complex influence of pro- and antioxidants on insulin sensitivity[2,3], we have chosen to explore the effects of catecholamines in human mature adipocytes.

Since we have performed our previous observations on rodent white fat cells[1] and since white adipocytes store energy in adipose depots, it was of utmost importance to verify whether our findings are relevant for human mature adipocytes. Indeed, white adipocytes are not found in human adipose tissue because it is yellow mature fat cells that are present in fat depots, regardless of their anatomical location. The yellow coloration found in humans is attributed to the storage of natural lipophilic pigments

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such as carotenoids, which are slowly metabolized. The white fat cells are somewhat specific of rodents, and this mere difference in the color is not the sole difference between fat cells from animal models and humans[4]. The sensitivity to vanadium regarding glucose accumulation in adipocytes is also different between rodents and humans, as recently reviewed[5]. Thus, an interspecies extrapolation step was mandatory prior to further development of our proposed combination of catecholamines plus vanadium for potential blood glucose-lowering approaches[1]. In the case of vanadium, its insulin-like properties originally described several decades ago[6,7] still requires the setting of novel administration forms to increase the benefit/risk ratio in diabetic patients[8,9].

The advantage of white adipocytes freshly isolated from young laboratory rodents previously used for the demonstration of the potential insulin mimicry of amines plus vanadium[1,10] is that such fat cells are highly sensitive to insulin stimulation of glucose metabolism and to the catecholamine stimulation of lipolysis. Interspecies comparative functional explorations of triacylglycerol synthesis (lipogenesis), triacylglycerol breakdown (lipolysis) and metabolite or adipokine release have shown multiple differences between rodent and human adipocytes, with human adipocytes being less metabolically active[11]. For example, the adrenergic stimulation of lipolysis is predominantly mediated by β_3-adrenergic receptor (β_3-AR) activation in rat and mouse, while it depends only on β_1-AR and β_2-AR activation in human adipocytes[4,12]. Similarly, the activation of α_2-ARs in human fat cells results in an antilipolytic response, which hardly occurs in rodent adipocytes since their equipment in α_2-ARs is rather limited[4]. Finally, the atrial natriuretic peptides are more lipolytic in human adipocytes than in the rodent ones[13,14].

All these considerations prompted us to test whether adrenaline and noradrenaline were activating glucose uptake in human adipocytes freshly isolated from pieces of abdominal subcutaneous adipose tissue removed during reconstructive surgery interventions in premenopausal women, as in[15,16]. When deciphering the effects of catecholamines in rat and mouse adipocytes, it has been evidenced that β-AR activation is not involved since catecholamines were able to activate glucose transport in fat cells from "beta-less" mice with triple knock-out of the subtypes of β-ARs[1]. In view of the above mentioned interspecies differences regarding adrenoreceptor equipment, it was necessary to perform such verification in human fat cells, and this implied the use of specific adrenergic agonists as reported in the following results.

Moreover, among the amines already reported to activate hexose uptake in human fat cells in the absence of insulin, it is worth mentioning benzylamine[17] and methylamine[15]. They are substrates of a copper-containing amine oxidase, the *AOC3* gene of which is highly expressed in human fat cells: the semicarbazide-sensitive amine oxidase (SSAO)[18], also known as primary amine oxidase[19], or vascular adhesion protein-1[20]. Benzylamine and methylamine have been included alongside insulin as positive controls of the hexose uptake activation in human adipocytes. As SSAO is not the sole amine oxidase present in adipocytes[21] [it coexists with monoamine oxidase (MAO-A, and to a lesser extend MAO-B)], their respective historical inhibitors semicarbazide and pargyline were also used. Also added in our control conditions was hydrogen peroxide, one of the reactive oxygen species (ROS) known to stimulate glucose uptake in fat cells[22] and is one of the historical insulin-mimetic compounds that act independently of insulin, while an excess of ROS hampers insulin action[3,23,24].

The following results, which can be considered as preclinical, will document that adrenaline and adrenaline stimulate hexose transport in human fat cells but in a manner that is not potentiated by sodium orthovanadate, not mimicked by β-AR or α_2-AR agonists and not hampered by SSAO and MAO inhibitors.

MATERIALS AND METHODS

Chemicals

(+/-)-Adrenaline (equivalent to epinephrine), (-)-noradrenaline (equivalent to norepinephrine), (-)-isoprenaline (equivalent to isoproterenol), dopamine, tyramine, benzylamine, sodium orthovanadate, collagenase A, human and bovine insulin and most of the other reagents were from Sigma-Aldrich-Merck (Saint Quentin Fallavier, France). [^{3}H]-2-deoxyglucose (2-DG) was from Perkin Elmer (Boston, MA, United States). The adrenergic agonists CL 316243 and BRL 37344 were given by Dr. Lafontan M. (Toulouse, France), while UK 14304 and RX 821002 were a gift from the late Dr. Paris H. (Toulouse, France).

Patients and adipose tissue surgery

Samples of abdominal subcutaneous adipose tissue were obtained with informed consent from a total of 34 women undergoing reconstructive surgery at the Department of Plastic Surgery (Rangueil Hospital, Toulouse, France). Mean age was 37 years (range: 18-59), and mean body mass index was 25.04 ± 0.65 kg/m^2 (range: 21-41). Adipose tissue samples were transferred to the laboratory in less than an hour after surgery and cut into small pieces then digested at 37 °C by collagenase under agitation. Preparations of buoyant adipocytes were obtained by filtration of the digested pieces through nylon stockings and two washes with Krebs–Ringer buffered at pH 7.5 with 15 mmol/L sodium bicarbonate, 10 mmol/L HEPES, supplemented with 3.5% of bovine serum albumin, as in[15]. No freezing/thawing sequences were inserted in the protocol for obtaining functional adipocytes, and 2-DG uptake assays were completed within 5 h after each surgical intervention. The study was in compliance with the INSERM guidelines and approved by the local ethics committee "Comité de Protection des Personnes Sud Ouest & Outre-Mer II" under the number DC-2014-2039.

Rodent adipocyte preparations

Male Wistar rats from Charles River (L'Arbresle, France) and mice of both genders from a mixed genetic background (129 Sv/ev, 129 Sv/J, FVB/N, C57BL/6J, and DBA/2) were housed in separate rooms at constant temperature (20-22 °C) and with a 12-h light-dark cycle. All the rodents had free access to food and water and were treated in accordance with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments)[25]. Only the mice that were considered as wildtype by Southern blot genotyping as described elsewhere[26] and were similar to those used as control for "β-less" mice in our previous study of catecholamine influence on adipocyte glucose transport[1] were euthanized after overnight fasting when 2- to 3-mo-old. Adipocyte preparations were obtained by collagenase digestion of perigonadic, retroperitoneal, perirenal and inguinal fat pads as previously described[1]. As for pieces of human adipose tissue, rodent fat pads were minced with scissors in Krebs-Ringer salt solution buffered at pH 7.5 and containing 3.5% fat-depleted bovine serum albumin.

Glucose transport assays

The only source of glucose for the cell preparations during glucose uptake assays was the non-metabolizable analogue [^{3}H]-2-DG, added at a final concentration of 0.1 mmol/L to fat cell suspensions as described previously[1]. Since the radioactive tracer (approximately 1300000 dpm/vial) was added for 10 min in the presence of fat cells after a 45 min preincubation period with the tested or reference agents, 2 mmol/L pyruvate was also present in the medium throughout the experiments for energy supply, as previously detailed[18]. Human or rodent fat cells were preincubated in 400 µL medium, then [^{3}H]-2-DG was added as 100 µL portions, and hexose uptake assays were stopped 10 min later with 100 µL of 100 µmol/L cytochalasin B. Then, 200 µL of cell suspension were immediately transferred to plastic centrifugation microtubes prefilled with dinonyl-phthalate (density 0.98 g/mL) before a 40 s spin to separate the buoying adipocytes from the medium as described previously[1,18]. The upper part of the tubes, containing radiolabeled hexose internalized in intact fat cells above the silicon layer was then counted in scintillation vials. The extracellular [^{3}H]-2-DG present in this upper part of the tubes, which was not internalized in cells, was determined with adipocytes whose transport activity was previously blocked by cytochalasin B at time 0. Though averaging 1%-5% of the radioactivity found in the upper phase, it was subtracted from all assays, as in[17]. Among the slight adaptations that differentiated assays with human fat cells from those with rat or mouse adipocytes was the use of human insulin instead of bovine insulin for rodent preparations and a higher richness in adipocytes: 20 mg lipids/400 µL instead of approximately 15 mg/400 µL.

Lipolytic activity determination

Glucose was present at 5.5 mmol/L in the Krebs-Ringer-based medium used for lipolysis assays, for which 2-DG and pyruvate were omitted, as already reported[15]. As above, tested agents were added to 400 µL of fat cell suspension at the start of a 90-min incubation at 37 °C under gentle shaking. Incubations were stopped on ice. Lipolysis was determined by using glycerol release as an index as already documented, considering that free fatty acid release exhibits parallel variations in our experimental conditions[16].

Statistical analysis

Results are presented as means ± SE of the mean of (*n*) observations. All the statistical analyses for comparisons between parameters used analysis of variance followed by post-hoc Dunnett's multiple comparisons test, analyzed with Prism 6 for Mac OS X (from GraphPad software, Inc). NS means non-significant difference.

RESULTS

Adrenaline and noradrenaline activate hexose transport in human adipocytes without need for vanadium

When incubation medium of human adipocytes was buffered at pH 7.5, no stimulation of basal hexose uptake was obtained with sodium orthovanadate at a final concentration of 100 μmol/L (Figure 1). Even the insulin-stimulated hexose transport, which was approximately three times higher than baseline, was not modified by 100 μmol/L vanadate. However, vanadium addition impressively potentiated the stimulatory effect of 1 mmol/L hydrogen peroxide on hexose uptake into human adipocytes (Figure 1), as already reported for rodent adipocytes[27]. Hydrogen peroxide was tested here as a reference because: (1) It stimulates hexose uptake in human adipocytes [17]; (2) Its action is potentiated by 100 μmol/L vanadate in rodent adipocytes[1]; and (3) It is one of the end-products of amine oxidase activity, regardless of the amine substrate or the type of amine oxidase activated[28].

All these control conditions confirmed our previous observations[15,17] and indicated that the human fat cell preparations were responsive to insulin regarding glucose transport activation. More importantly, the synergism between vanadium and hydrogen peroxide was in line with the characterization of the insulin-like properties of peroxovanadate, the compound generated by the combination of vanadate and hydrogen peroxide[5,29].

Figure 1 also shows that vanadate did not modify the effect of 100 μmol/L benzylamine, which elicited a stimulation that was equivalent to approximately one-third of the maximal insulin stimulation. Similarly, the stimulatory effect of adrenaline and noradrenaline on hexose uptake in human fat cells was not enhanced by vanadate (Figure 1). This was strikingly different from the synergism found between vanadate and amines regarding activation of glucose uptake in rodent adipocytes[1,27].

Together, these first observations indicated that high doses of adrenaline and noradrenaline can acutely activate glucose transport in human fat cells, at least when incubated with the cells at 100 μmol/L for 45 min. The unexpected difference when compared with animal models was that the 'insulin-like' effect of the amines was not enhanced by vanadium in human fat cells. In other words, catecholamines stimulated 2-DG uptake in human adipocytes without the need for vanadium. This capacity to enhance glucose transport deserved further study since it could constitute a novel rationale for increasing glucose consumption in peripheral tissues. We investigated whether other amines could activate 2-DG uptake in human adipocytes, either in a vanadium-dependent or independent manner. To this aim, we compared the responses of rat and human adipocytes.

Comparative study of the glucose transport stimulation by various amines in the absence and the presence of vanadium

Figure 2 shows that the behavior of human adipocytes was clearly different from that of rat adipocytes regarding the synergism between vanadium and amines. The clear potentiation occurring between vanadate and most of the tested amines, already evidenced in rat adipocytes[1], could not be merely extrapolated to human adipocytes. However, this interspecies comparative approach indicated that adrenaline and noradrenaline were the most powerful agents among the fifteen biogenic amines tested in human adipocytes and demonstrated that not any given amine was able to activate glucose uptake at 1 mmol/L. For unknown reasons, the relative rank order of potency for (either cyclic or aliphatic) amines activating hexose uptake was not the same in rat and human adipocytes. Another important finding drawn from this comparison is that the lack of potentiation between vanadium and amines was generalized to all the amines tested on glucose transport in human adipocytes, at least under our experimental conditions.

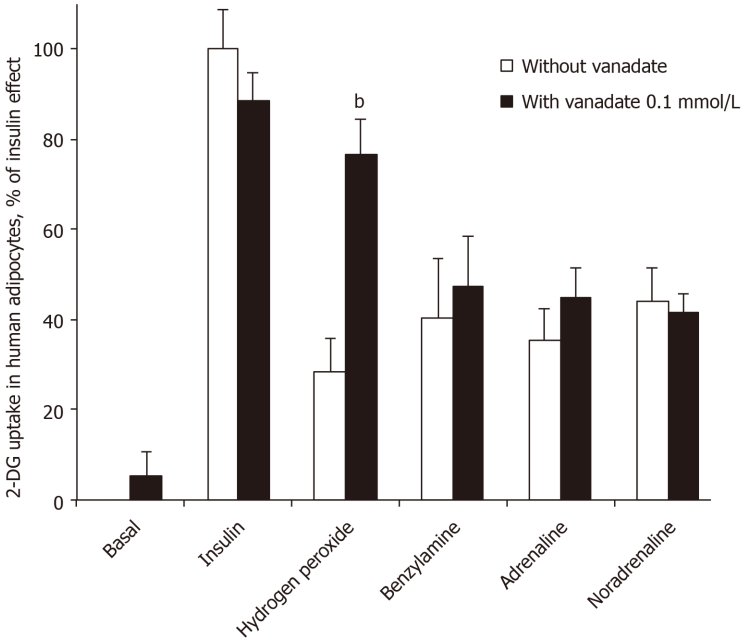

Figure 1 Influence of vanadate on glucose transport in human adipocytes in response to insulin, hydrogen peroxide and naturally occurring amines. Suspensions of human fat cells were preincubated without (white columns) or with 100 μmol/L sodium orthovanadate (dark columns) for 45 min in the presence of the indicated amines at 100 μmol/L or with 100 nmol/L insulin and hydrogen peroxide at 1 mmol/L. [^{3}H]-2-deoxyglucose uptake assay was then immediately performed for 10 min and expressed as the percentage of maximal stimulation by insulin (set at 100%), with baseline set at 0% (the respective levels of which were: 1.29 ± 0.12 and 0.45 ± 0.04 transported nmol 2-deoxyglucose/100 mg cell lipids/10 min). Each column is the mean ± standard error of the mean from 7 to 10 determinations. In all conditions tested, 2-deoxyglucose uptake was significantly different from basal. A significant influence of vanadium when compared to respective control without vanadate was observed at: $^bP < 0.01$. 2-DG: 2-Deoxyglucose.

Comparative study of methylamine-dependent stimulation of glucose transport in rat and human adipocytes

Methylamine, which does not contain any benzene or catechol ring in its chemical structure and is the simplest molecule well-recognized as an SSAO substrate, was then used for further comparison between rat and human adipocytes. The methylamine stimulation of 2-DG uptake, which occurred only in the presence of 100 μmol/L vanadate in rat adipocytes, was entirely abolished by 1 mmol/L semicarbazide, the reference inhibitor of SSAO, while it was partially resistant to the MAO inhibitor pargyline, even when present at 1 mmol/L (Table 1). In human adipocytes, methylamine activated 2-DG uptake without the need for vanadium. The same pattern of inhibition was observed in both species, albeit the glucose uptake activity exhibited lower amplitude in humans (Table 1). Such interspecific difference in the magnitude of the response was rather expected since it has been well established that human adipocytes are less metabolically active than rodent fat cells. This did not prevent drawing of the same conclusion for both models: the combination of pargyline and semicarbazide largely impaired the methylamine activation of hexose transport (Table 1).

Thus, in rats and humans, semicarbazide plus pargyline likely impaired the release of oxidation products during methylamine catabolism by SSAO and/or MAO, and this consequently prevented methylamine to activate 2-DG uptake. It can be postulated that activation of the amine oxidases expressed in adipocytes was supporting the 2-DG uptake activation in response to millimolar doses of methylamine. Hydrogen peroxide, one of the products generated during oxidative deamination was supposed to be involved in this hexose uptake stimulation, according to previous studies in adipocytes[1] or cardiomyocytes[30]. However, this paradigm does not address the different sensitivities of hydrogen peroxide and amines regarding potentiation by vanadium in human fat cells. Additional investigations were therefore required to depict the mechanisms underlying the stimulatory action of catecholamines on the glucose entry into human adipocytes. It was decided to search whether degradation products other than hydrogen peroxide could mediate the catecholamine effect on 2-DG uptake by further comparing rodent and human adipocytes.

Table 1 Methylamine stimulation of hexose uptake in rat and human adipocytes is dependent on semicarbazide-sensitive amine oxidase

	Rat adipocytes with vanadate 0.1 mmol/L	Human adipocytes without vanadium
Incubation condition	2-DG uptake (nmol/100 mg lipids/10 min)	
Control	1.30 ± 0.12	0.49 ± 0.05
Insulin 100 nmol/L	13.08 ± 0.31[e]	1.49 ± 0.19[e]
Methylamine 1 mmol/L	11.27 ± 1.17[e]	0.86 ± 1.3[b]
Met + pargyline 1 mmol/L	6.37 ± 0.88[f]	0.84 ± 0.09
Met + semicarbazide 1 mmol/L	1.32 ± 0.19[f]	0.60 ± 0.08[c]
Met + pargyline + semicarbazide	1.18 ± 0.34[f]	0.59 ± 0.07[d]

Glucose uptake was assayed for 10 min after 45-min incubation with the indicated doses of agents. Mean ± SE of the mean of 8-9 rat and 18 human adipocyte preparations. Difference between insulin or methylamine and control significant at:
[a]$P < 0.05$.
[b]$P < 0.01$.
[e]$P < 0.001$.
Significantly different from methylamine alone at:
[c]$P < 0.05$.
[d]$P < 0.01$.
[f]$P < 0.001$.
2-DG: 2-Deoxyglucose; Met: Methylamine.

Effects of waste metabolites of catecholamine catabolism on glucose transport in mouse and human adipocytes

Since the reaction end-products of benzylamine oxidation by amine oxidases, benzaldehyde and ammonia, have been found to be inactive on glucose transport in human adipocytes[18], they were not further investigated here. By contrast, pyrocatechol and benzoquinone, which can be considered as final metabolites of catecholamine catabolism, have never been tested on glucose transport, at least to our knowledge. Thus, it was investigated how their putative effects could be improved by vanadate. The metabolite pyrocatechol, formed by a benzene core carrying two hydroxyl substituents, was inefficient on glucose transport in both mouse and human adipocytes when tested alone from 1 μmol/L up to 1 mmol/L (Figure 3). Pyrocatechol was also unable to activate hexose uptake in rat adipocytes (not shown). Even when tested with vanadate, pyrocatechol was inefficient in the three species, with only a tendency to generate higher uptake levels in mice, without reaching significance and with largely weaker magnitude than insulin stimulation (Figure 3).

In many biological materials, the oxidation of catechol gives reddish-brown melanoid pigments, derivatives of benzoquinone. We therefore tested benzoquinone on glucose transport. In mouse adipocytes that were highly responsive to insulin, benzoquinone did not notably activate 2-DG uptake, with or without vanadium (Figure 3). Benzoquinone even inhibited basal 2-DG uptake at 1 mmol/L, and a similar pattern was observed in human adipocytes (Figure 3). Apparently, these "waste" products of catecholamine catabolism were not responsible for the mild activation of hexose uptake by high doses of (nor)adrenaline either in rodent[1] or human adipocytes (see Figures 1 and 2). Moreover, benzoquinone was inhibitory at millimolar doses.

An additional investigation was performed with pyrocatechol and benzoquinone on mouse adipocytes and indicated that they were neither able to activate lipolysis as did adrenaline or adrenaline (Figure 4) nor able to impair the lipolytic effect of the catecholamines when tested at 1 μmol/L (not shown). Hence, these waste products cannot be suspected to impair or to support the effects of (nor)adrenaline on glucose entry in human fat cells.

During all these verifications, the sole 2-DG uptake activation demonstrated to depend on amine oxidase activity was that of methylamine. Thus, verifying whether the effects of (nor)adrenaline were sensitive to blockade by pargyline and semicarbazide in human adipocytes remained mandatory.

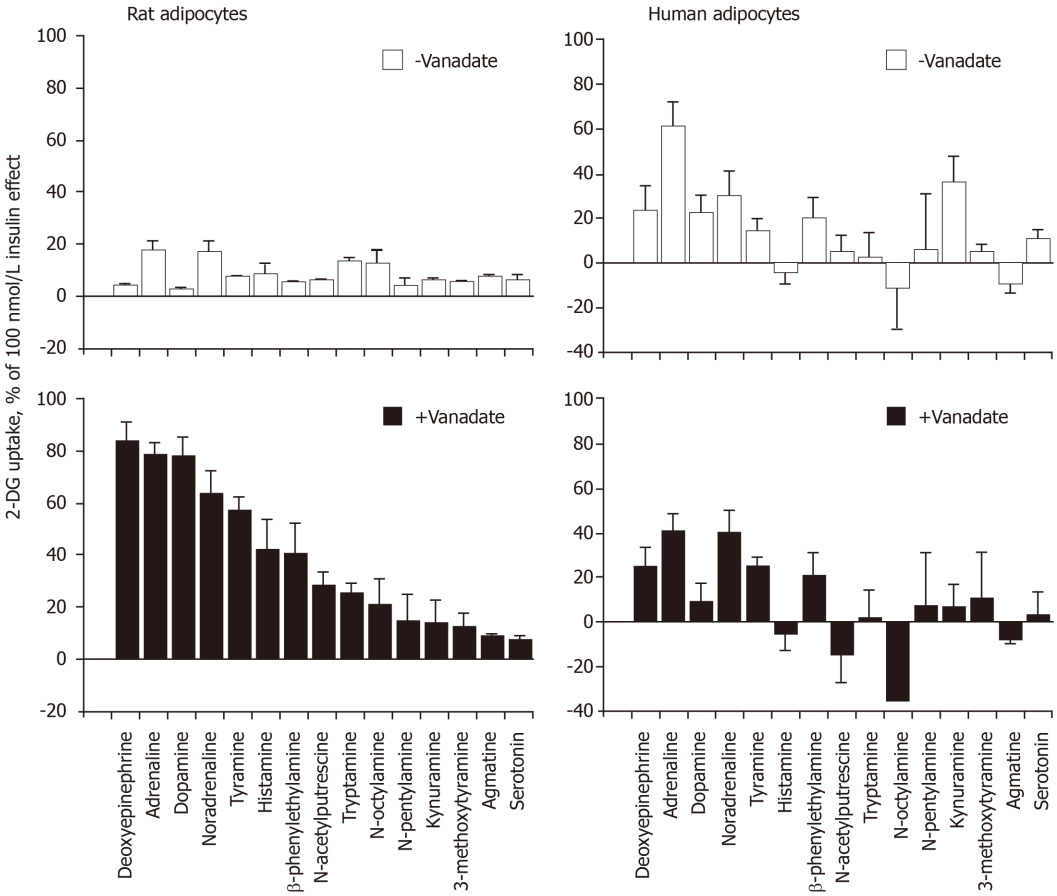

Figure 2 Interspecific differences between rat and human adipocytes in hexose uptake activation by various amines with and without vanadium. Rat (left) or human (right) fat cells were incubated with 1 mmol/L of the indicated amines in the absence (upper panels, open columns) or the presence of 0.1 mmol/L sodium orthovanadate (lower panels, black columns) just before 2-deoxyglucose uptake assays. Hexose uptake was expressed as the percentage of maximal stimulation induced by 100 nmol/L insulin (set at 100%, with basal uptake set at 0). Negative percentages traduced a transport that was lower than baseline. Each column is the mean ± standard error of the mean of 4 to 11 experiments in rat adipocytes and of 3 to 12 preparations of human adipocytes and is presented according to the decreasing rank order of amine-induced stimulation obtained in rat adipocytes when tested at 1 mmol/L + 0.1 mmol/L vanadate, according to our previously published data[1], redrawn here in the left panel with the author's permission. 2-DG: 2-Deoxyglucose.

The activation of hexose uptake in human adipocytes by noradrenaline and adrenaline resists inhibition by pargyline and semicarbazide

The glucose transport in human adipocytes incubated with 100 nmol/L insulin was defined as the maximal activation of 2-DG uptake and set at 100%. Basal and insulin-stimulated 2-DG uptake resisted the blockade by the combination of amine oxidase inhibitors: pargyline + semicarbazide (parg + semi) (Figure 5A). Nevertheless, as above with methylamine, the use of benzylamine and octopamine confirmed that 'classical' amine oxidase substrates were able to activate glucose entry in human fat cells in a manner that was abolished by parg + semi (Figure 5A). Surprisingly, this was not the case for the activation of 2-DG uptake by 0.1 and 1 mmol/L of noradrenaline and adrenaline, which was not impaired by parg + semi, ruling out the contribution of amine oxidase-dependent oxidation (Figure 5B and C). When tested separately, pargyline and semicarbazide were unable, even at 1 mmol/L to inhibit the adrenaline-induced hexose transport (respective 2-DG uptake levels were in nmol/100 mg lipid/10 min: adrenaline, 1.01 ± 0.12; adrenaline + semicarbazide, 1.01 ± 0.17; adrenaline + pargyline , 0.95 ± 0.10; n = 16; NS, not shown).

Being resistant to parg + semi, the stimulatory action of catecholamines on glucose entry in human adipocytes was not dependent on amine oxidase activity as observed in rodents. Albeit we recently ruled out the contribution of β-AR stimulation in the effects of catecholamines plus vanadium on glucose transport in rodent adipocytes[1], it became necessary to explore this putative mechanism in human adipocytes.

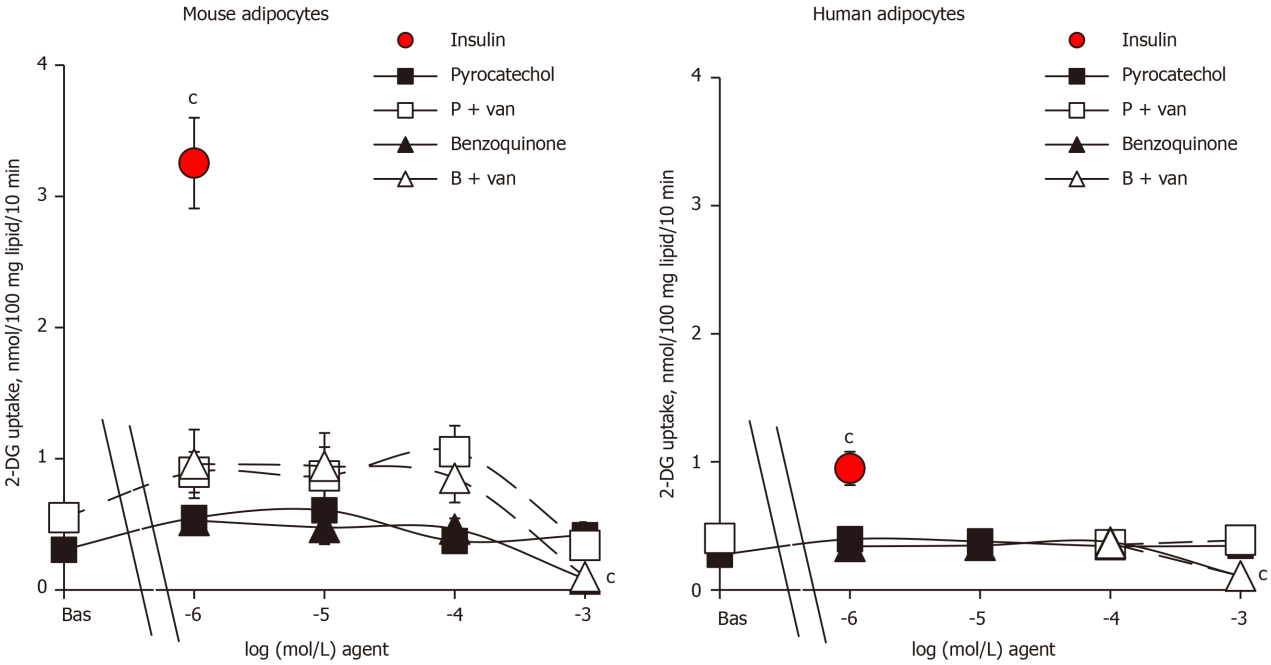

Figure 3 Lack of stimulatory influence of pyrocatechol and benzoquinone on hexose uptake in mouse and human adipocytes. Mouse (left panel) and human (right panel) fat cells were incubated for 45 min with increasing concentrations (from 1 µmol/L to 1 mmol/L, indicated as log of molar concentration) of pyrocatechol (squares) or benzoquinone (triangles) without (closed symbols) or with 100 µmol/L vanadate (open symbols) just before assaying [^{3}H]-2-deoxyglucose uptake for a 10-min period. Basal (without any agent added) and maximal hexose uptake in response to 1 µmol/L insulin (red circle, bovine hormone for mouse adipocytes and human recombinant protein for human adipocytes) are given with the same Y-axis scale for the sake of comparison. Mean ± standard error of the mean of 9 and 7 separate experiments for mouse and human adipocyte preparations containing 15 ± 2 and 20 ± 3 mg lipid/400 µL assay tube, respectively. Difference from basal uptake was significant at: $^cP < 0.001$. 2-DG: 2-Deoxyglucose; B: Benzoquinone; van: Vanadate; P: Pyrocatechol; Bas: Basal.

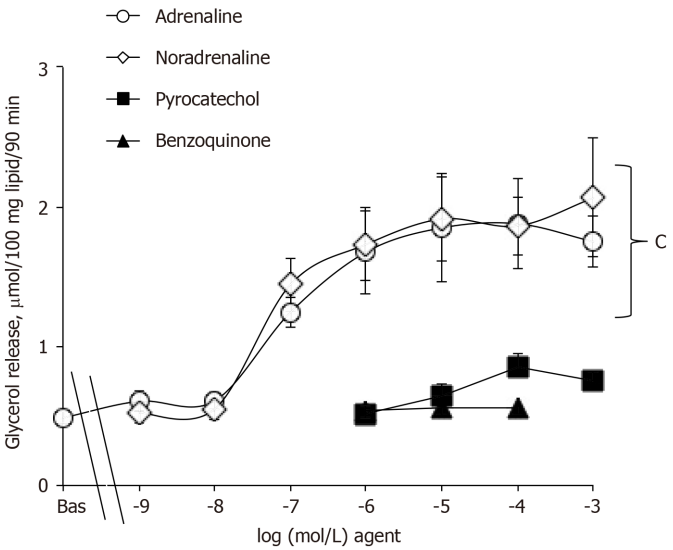

Figure 4 Dose-dependent activation of lipolysis by adrenaline and noradrenaline but not pyrocatechol and benzoquinone in mouse adipocytes. Glycerol release was assessed after 90 min incubation of mouse adipocytes without (basal) or with increasing concentrations of adrenaline (open circles), noradrenaline (open diamonds), pyrocatechol (black squares) or benzoquinone (dark triangles). Each condition is the mean ± standard error of the mean of 5-6 adipocyte preparations. The doses of adrenaline and noradrenaline ranging between 1 µmol/L and 1 mmol/L induced a response different from basal at: $^cP < 0.001$. Bas: Basal.

Is there a direct activation of hexose uptake in human adipocytes by adrenoreceptor agonists or a blockade by adrenergic antagonists?

Figure 6 shows that the glucose transport of human adipocyte preparations that were responsive to human insulin could not be activated notably by any of the five β-adrenergic receptor agonists tested. The α_2-AR agonist UK 14304 (also known as brimonidine) was also inefficient, arguing that α_2-adrenergic activation does not

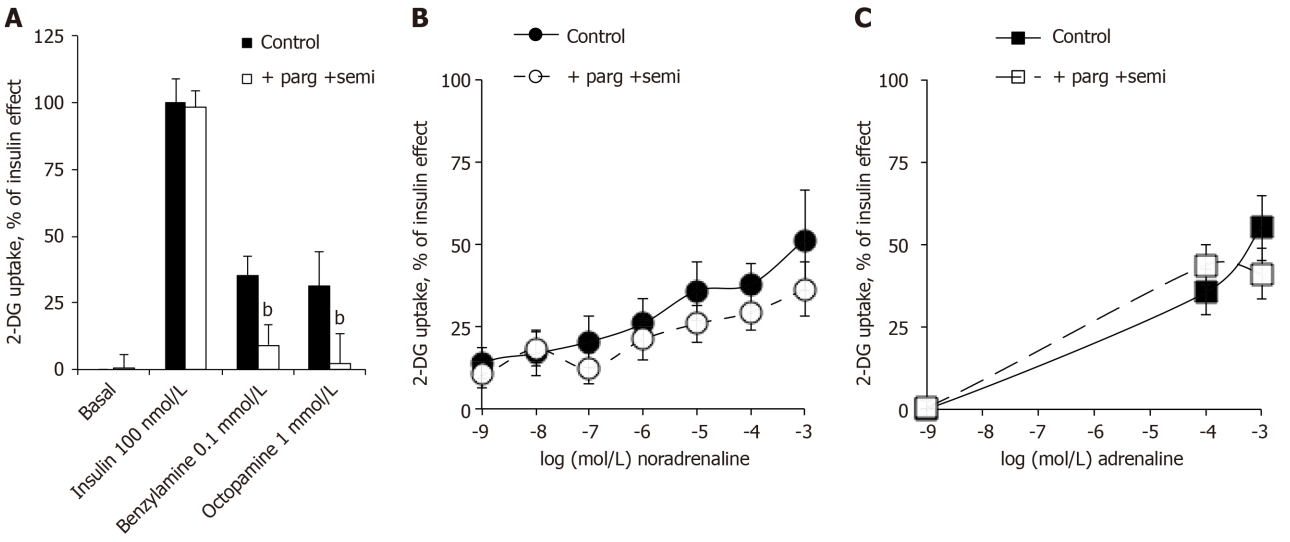

Figure 5 Inhibition by pargyline and semicarbazide of benzylamine and octopamine effects on hexose uptake in human adipocytes but not of noradrenaline and adrenaline effects. Human fat cells were incubated in the presence of the indicated agents without (control, black symbols) and with the combination of 100 µmol/L pargyline plus 1 mmol/L semicarbazide (open symbols) before being subjected to 2-deoxyglucose uptake assay. A: Insulin, benzylamine and octopamine: mean ± standard error of the mean of 7 adipocyte preparations. A significant inhibition when compared to respective control was observed at: $^{b}P < 0.01$. B: Increasing doses of noradrenaline: mean ± standard error of the mean of 13 cases. C: Indicated doses of adrenaline: mean ± standard error of the mean of 17 cases. No significant difference was found between inhibitor and respective control conditions. 2-DG: 2-Deoxyglucose; parg: Pargyline; semi: Semicarbazide.

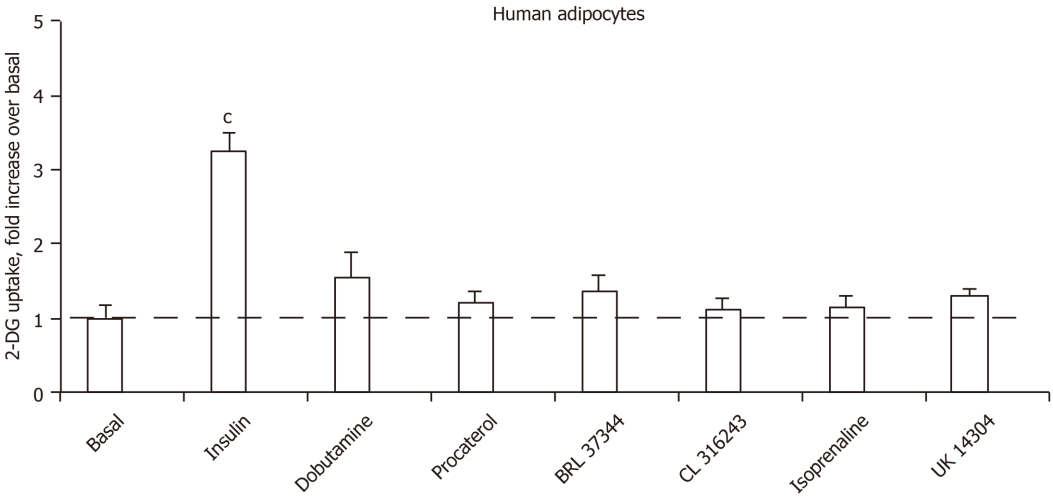

Figure 6 Influence of insulin, β- and α-adrenergic receptor agonists on hexose transport in human adipocytes. 2-Deoxyglucose uptake assay was performed without (basal) and with 100 nmol/L insulin or 1 µmol/L of the indicated adrenergic receptor agonists. Dobutamine: β_1-AR agonist; procaterol: β_2-AR agonist; BRL 37344 and CL 316243: β_3-AR agonists; isoprenaline: pan-agonist of the three subtypes of β-ARs; UK 14304: α_2-AR agonist. Glucose transport was expressed as fold increase relative to basal uptake set at 1.0 (dotted line). Each column is the mean ± standard error of the mean of 9-12 individual adipocyte preparations. Only insulin-induced uptake was significantly different from baseline at: $^{c}P < 0.001$. 2-DG: 2-Deoxyglucose.

enhance glucose uptake (Figure 6). Of note, the tested β_1- and β_2-adrenergic agonists were active at 1 µmol/L on lipolysis activation in human fat cells (or in provoking antilipolytic response in the case of UK 14304), as previously reported in independent studies[31,32].

In additional experiments performed to study the sensitivity to antagonists, RX 821002 was chosen for blocking α_2-ARs, and the pan-antagonist bupranolol for blocking the β-ARs. Again, adrenaline at 100 µmol/L increased the basal 2-DG uptake (basal: 0.34 ± 0.02, adrenaline: 0.66 ± 0.04; $n = 4$; $P < 0.01$), and this was not impaired by 10 µmol/L of each of the antagonists (adrenaline + RX 821002: 0.61 ± 0.06; adrenaline + bupranolol: 0.58 ± 0.04 nmol 2-DG transported/100 mg lipids/10 min; $n = 4$; NS, not shown).

Thus, the use of adrenergic agents could not mimic or block the stimulatory effect of noradrenaline and adrenaline on hexose uptake. At this stage, the role of autoxidation products of the catecholamines was investigated. Alongside adrenochrome and noradrenochrome, the chemistry of catecholamine degradation encompasses numerous ROS, aldehydic molecules and oligomeres implied in neurotoxicity[33]. Rather than testing these highly reactive intermediates, which are rather unstable, it was investigated whether the relatively short-term effect of millimolar doses of adrenaline could be prevented by antioxidant pretreatment. As hydrogen peroxide is active on hexose uptake in adipocytes, it was verified whether its generation was prevented by catalase. Figure 7 shows that catalase impaired the adrenaline-induced stimulation of 2-DG uptake in human adipocytes. The addition of glutathione, expected to limit hydrogen peroxide dismutation by catalase, could not reach complete blockade of adrenaline effect. Lastly, the phosphoinositide 3-kinase inhibitor wortmannin was able at 1 μmol/L to inhibit the effect of adrenaline as well as that of insulin (Figure 7), suggesting that in both cases the activation of hexose uptake was due to a phosphoinositide 3-kinase/protein kinase B-induced glucose carrier recruitment to the cell surface.

DISCUSSION

In a recent study, in view of the powerful stimulating effect of the combination of catecholamines plus sodium orthovanadate on glucose transport in rodent adipocytes, we have proposed that the use of catecholamines might improve the antidiabetic effect of vanadium by reducing its efficient therapeutic doses and by lowering its toxicity[1]. In the present study, we aimed to extrapolate to human adipocytes the description of the insulin-like nature of the synergism between catecholamines and vanadium on glucose utilization. The results of our present human study clearly indicate that adrenaline and noradrenaline activate hexose uptake in human fat cells at doses comprised between 0.1 and 1 mmol/L. In fact, human fat cells respond to catecholamine exposure for 45 min by a stimulation of hexose uptake that represents one-third to one-half of the maximal response to insulin, depending on the individuals. To our knowledge, it is the first time that such a short-term, non-negligible, insulin-like effect of these two naturally occurring catecholamines is observed in human fat cells. However, this stimulation was not further enhanced by the presence of vanadium and never reached the 80%-90% of the maximal insulin-dependent stimulation of glucose uptake, as it was observed in rodents[1]. In other words, the synergism found between (nor)adrenaline and sodium orthovanadate in rat fat cells was not observed in human adipocytes. This interspecific difference, already observed for the sensitivity to decavanadate[5], abruptly ceased our proposal to use catecholamine derivatives in future strategies aimed at improving the benefit/risk ratio of vanadium-based antidiabetic treatments.

However, a potentiation of the mild activation effect of hydrogen peroxide with 0.1 mmol/L vanadate (a dose inefficient on its own to activate 2-DG uptake) occurred in both human and rodent adipocytes (Figure 1 and[1]). It is not the synergism between hydrogen peroxide and vanadium, which generates peroxovanadate, a powerful insulin-mimicking agent on glucose utilization, that was primarily involved in such unexpected interspecific differences. Curiously, in the same experimental conditions, catecholamines were not the sole amines that behaved differently between human and rodents fat cells. The widely recognized SSAO substrates, benzylamine and methylamine, and various other biogenic amines did not exhibit any synergism with vanadium in activating 2-DG uptake in human fat cells, while most of them were potentiated in rodent adipocytes[1,10,34].

Other unexpected differences between rat and human adipocytes did not facilitate a mere extrapolation of our previous findings regarding the synergism between catecholamines and vanadium. For instance, dopamine, which was as stimulatory as adrenaline and noradrenaline in rat adipocytes[1], was not active in human fat cells. Similarly, deoxyepinephrine was much more active on glucose uptake in rats than in humans. Unfortunately, we cannot provide at the moment any explanation for such differences, and this also applies for the rank order of potency for the various amines tested without and with vanadate on hexose uptake, since that found in the rat model is not at all predictive of that found in humans. The catabolism of the biogenic amines and the fates of the vanadate/vanadyl forms of the metal ion are probably different in the two species, as it is also the case for hydrogen peroxide generation/catabolism.

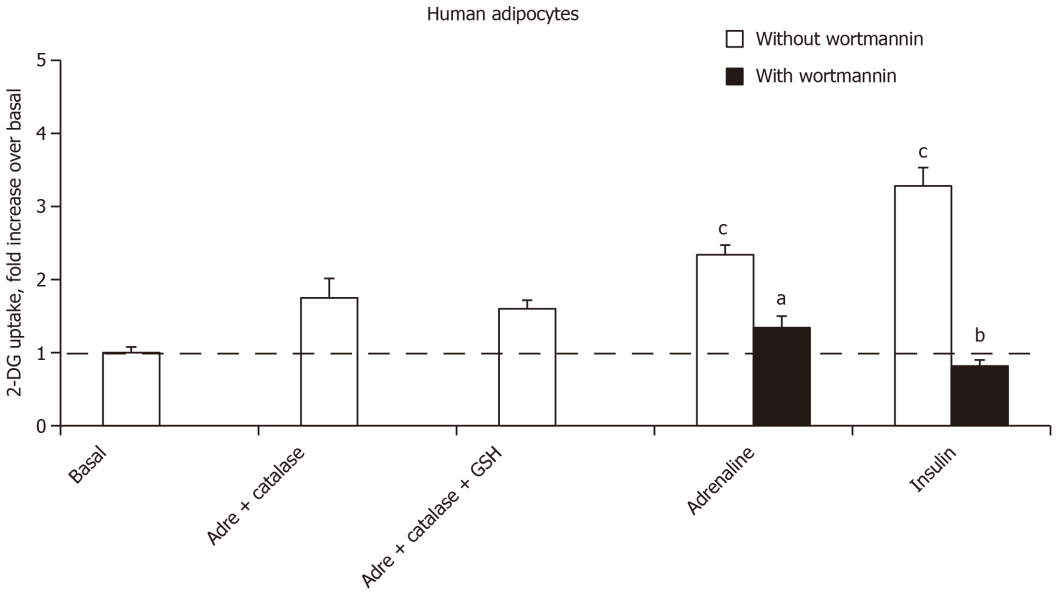

Figure 7 Inhibition of adrenaline-induced hexose uptake in human adipocytes by catalase, glutathione and wortmannin. The pretreatment of adipocytes by catalase alone (5000 IU/mL) or in combination with 1 mmol/L glutathione was started 10 min before the addition of 1 mmol/L adrenaline, which was incubated for 45 min before performing 2-deoxyglucose uptake assay for 10 min. The mean ± standard error of the mean of 4 to 6 separate experiments is given as fold increase above basal 2-deoxyglucose uptake. A significant difference from basal was observed at: $^{c}P < 0.001$; Different from 1 mmol/L adrenaline alone at: $^{a}P < 0.05$, or from 100 nmol/L insulin alone at: $^{b}P < 0.001$. 2-DG: 2-Deoxyglucose; adre: Adrenaline.

Nonetheless, several common features were observed in this comparative approach: first, no significant effect of serotonin was evidenced in both species; then, the same dose of vanadate that was inefficient on its own on basal or insulin-stimulated hexose uptake, *i.e.*, 0.1 mmol/L, potentiated the hydrogen peroxide effect in both species. Other points of resemblance between rodent and human adipocytes are discussed below, but it must be kept in mind that the essential difference between the two models is that rat fat cells are definitely more metabolic active than the human ones, as attested by the absolute values of maximal hexose transport in response to insulin (see Figure 3 and Table 1).

Although impressed by the distinct intrinsic activity and vanadium sensitivity of the fifteen amines tested, we further attempted to decipher the mechanisms of action implied in the two most active on glucose uptake in human adipocytes: adrenaline and noradrenaline. As with rodent adipocytes, adrenaline and noradrenaline behaved differently from typical SSAO substrates (benzylamine, methylamine): only the latter were not able to activate glucose uptake in the presence of semicarbazide, alone or combined with the monoamine oxidase inhibitor pargyline. In this aspect, human adipocytes resemble the rat ones. Nevertheless, the lack of inhibition of (nor)-adrenaline effect by the combination parg + semi is puzzling since catecholamines are well-known substrates of MAO, abundant in adipocytes[27].

We used the combination of MAO and SSAO inhibitors in the present study since both MAO and SSAO substrates are able to mimic insulin-like effects in adipocyte models[27], and since methylamine is a product of adrenaline oxidation by MAO together with hydrogen peroxide. In turn, methylamine is a substrate for SSAO, also generating hydrogen peroxide[35]. This postulated two-step process could explain why adrenaline was the most powerful among the amines tested in stimulating glucose uptake in human adipocytes. But it cannot explain why the adrenaline effect was so weakly impaired by parg + semi combination, capable to block the methylamine-induced glucose transport. Moreover, semicarbazide, although inactive on MAO, inhibits the activity of the enzymes encoded by the *AOC1* and *AOC3* genes (diamine oxidase and SSAO) and other related copper containing amine oxidases[36]. Despite its similitude with our previous observations in rodent adipocytes[1], the resistance to the blockade by the parg + semi combination remains a characteristic of the effects of adrenaline and noradrenaline that is not totally elucidated. Rather than testing the presence of other putative amine oxidases that could be implied in the oxidation of adrenaline and noradrenaline, we explored possible transduction signals other than the amine oxidase-mediated pathway.

The absence of plateau in the dose-response curve to noradrenaline activation of 2-DG uptake in human fat cells was somewhat indicative that the mechanism involved is not mediated by a single receptor activation. Indeed, the linear increase of uptake in response to noradrenaline from 1 nmol/L to 1 mmol/L found in human adipocytes clearly contrasted with the typical sigmoid curve seen in mouse adipocytes when the adrenergic stimulation of glycerol release was determined. Only the latter response corresponds to a classical activation of the lipolytic cascade, implying an amplification system with successive activation of β-AR/Gs protein/adenylyl cyclase/protein kinase A/lipases (compare Figures 4 and 5). In keeping with this, none of the various adrenergic agonists tested was able to activate 2-DG uptake in human adipocytes, and the effect of adrenaline was insensitive to the α_2- and β-AR antagonists used. These results were in perfect agreement with our recent report showing that β-AR or α_2-adrenergic receptor stimulation was not involved in the stimulation by catecholamines plus vanadium of glucose transport in rodent adipocytes[1]. Our pharmacological approach still leaves open a putative mediation of the glucose transport stimulation by α_1-AR activation, as proposed in a clinical study based on the effect of noradrenaline and the α_1-AR agonist norfenefrine during microdialysis experiments in obese patients [37]. When keeping in mind that neither α_1-AR agonist nor α_1-AR antagonist modified 2-DG uptake in rat fat cells[1], such α_1-AR contribution does not appear plausible and cannot be the sole mechanism supporting the glucose uptake stimulation by 100 μmol/L noradrenaline or adrenaline. Even the stimulation of glucose uptake in rat cardiomyocytes by the recognized α_1-AR agonist phenylephrine has been reported to be biphasic: mediated partly by calcium release and by hydrogen peroxide[38].

It was then the products of catecholamine autoxidation that were suspected to produce activation of hexose uptake in view of: (1) The lack of classical sigmoid shape of the dose-response curve to adrenaline; (2) The resistance to amine oxidase inhibitors, although some hydrazine derivatives have been proven to limit the lipid oxidation by reactive carbonyl compounds[39]; and (3) The impairment caused by antioxidants on the activation by adrenaline + vanadate in rodent adipocytes[1].

The autoxidation of (nor)adrenaline, which generates (nor)adrenochrome and known to be increased by metal ions, can be delayed by EDTA or pH acidification. These two conditions have not been tested in the present study since they directly interfere with glucose transport activity. Although we did not assess whether the presence of vanadium was increasing adrenochrome generation in adipocyte preparations, we did not note any dark coloration in the incubation tubes under any condition. In addition, it must be repeated here that the addition of vanadium to adipocyte incubation medium did not increase the catecholamine-stimulated hexose uptake in human fat cells. However, we were aware that sodium vanadate can elicit pH alkalinization and thereby hexose uptake stimulation. For this reason, we prevented any pH elevation by 0.1 mmol/L vanadate owing to the strongly buffered incubation medium we used. Thus, the putative contribution of adrenochrome in the observed effects is not dealing with the lack of potentiation of adrenaline-induced uptake by vanadate since it has been reported that vanadate enhances the *in vitro* formation of adrenochrome from epinephrine, alongside a reduction of antioxidative defenses, a property that might be linked to vanadate toxic effects in various cell types [40,41]. The fact that the adrenaline stimulation of glucose transport was limited by catalase treatment is another element for discarding the involvement of adrenochrome. Nevertheless, its putative role remains to be definitely ruled out.

One of the limitations in our approach is that we cannot depict the signal transduction elicited by catecholamines when partially mimicking the insulin stimulation of glucose transport. Although we tested two among the numerous metabolites of catecholamines, we did not pay attention to the transient and highly reactive aldehydic molecules generated during either autoxidation or during catabolism by MAO and catechol-O-methyltransferase[33]. Moreover, we did not determine whether there was an appearance of the quinones that are produced during the degradation of (nor)adrenaline into (nor)adrenochrome[42]. However, in accordance with the cytotoxicity of these products, the millimolar dose of benzoquinone has been found to abolish transport activity in adipocytes. While various quinones probably occurred with dopamine also, they did not elicit a detectable effect on 2-DG uptake. Thus, the quinone-based toxic metabolites do not seem to support the catecholamine effect.

It cannot be excluded that others of the numerous metabolites of catecholamine degradation are involved in the *in vitro* effect we detected, but the participation of hydrogen peroxide, endowed with insulin-like effects, could not be clearly evidenced in our experiments, excepted by catalase treatment. Catalase and glutathione were used since they have been shown to protect neuroblastoma cells against the

cytotoxicity of dopamine, due to oxidative stress by generating excessive ROS *via* MAO-catalyzed oxidative deamination and *via* autoxidation[43]. On the contrary, ascorbic acid has been reported to be unable to prevent the autoxidation of catecholamines that occurs readily in the oxygen-saturated incubation media of *in vitro* experiments[44,45]. At last, the inhibition by wortmannin allowed postulating that high doses of catecholamines were activating the recruitment of glucose transporters at the surface of human fat cells.

Finally, noradrenaline and adrenaline are vasoconstrictor agents that stimulate cardiac inotropism, strongly elevate blood pressure as well as increase blood glucose in order to better respond to stress conditions by a behavior well-known from invertebrates to vertebrates as the "fight or flight" response. It is not so astonishing to observe that at high doses these catecholamines are able to activate the glucose utilization in cells in order to facilitate energy consumption. Although the adipocytes are specialized for releasing their lipid stores when the organism requires energy supply, they have to increase glucose uptake/consumption at the same time to perform fatty acid re-esterification to avoid excessive lipolysis. The simultaneous activation of lipolysis and the enhancement of other metabolic pathways such as lipogenesis of fatty acid oxidation is therefore physiologically relevant under adrenergic activation and seems to occur in both animal and human adipocytes. It is the potentiation of the somewhat "insulin-mimicking" properties of catecholamines that does not occur with vanadium in human adipocytes. This does not preclude the interest of the current improvements of the antidiabetic therapeutic applications of vanadium[9,46,47] but seriously limits the relevance of the observations made on rat adipocytes[1,27,48] regarding the promising insulin mimicry of vanadium compounds.

CONCLUSION

This preclinical study describes *in vitro* the activation of hexose uptake in human adipocytes by high doses of catecholamines. It also demonstrates that this insulin mimicry has no interest for improving the benefit/risk ratio of vanadium-based antidiabetic complexes since there is no synergism between catecholamines and vanadate regarding glucose uptake in isolated human adipocytes. Moreover the puzzling effect of catecholamines is not entirely mediated by adrenoreceptor stimulation or by MAO- and SSAO-dependent amine oxidation. As lower doses of catecholamines are recognized to rise blood pressure and blood glucose *in vivo*, no therapeutic use of the present observations can be postulated at the present time.

ARTICLE HIGHLIGHTS

Research background

We have recently reported a synergism between vanadium and catecholamines that generates a powerful activation of glucose transport in rodent adipose cells. Since the combination vanadium/adrenaline or vanadium/noradrenaline mimicked insulin activation of glucose handling in a manner depending on the production of reactive oxygen species, we proposed that further research on vanadate/catecholamine complexes could develop novel, less toxic antidiabetic therapeutic approaches for vanadium compounds.

Research motivation

To extrapolate to humans the potential antihyperglycemic properties of the vanadate/catecholamine combination found in animal models, we aimed to verify whether several amines, including adrenaline and noradrenaline, were able together with vanadate to reproduce the insulin-induced stimulation of glucose transport into human adipocytes.

Research objectives

To evaluate the impact of various biogenic amines, including the well-known catecholamines, adrenaline and noradrenaline, without and with vanadium, on glucose transport in human adipose cells.

Research methods

Preparations of freshly isolated human adipocytes, obtained from patients undergoing plastic surgery, were subjected to a pharmacological exploration of glucose transport owing to short-term uptake assays performed with the non-metabolizable radiolabeled analogue 2-deoxyglucose. An interspecies approach compared the responses of rat, mouse and human adipocytes subjected to similar stimuli.

Research results

In human adipose cells, the stimulation of glucose transport by insulin increased by two-to three times the basal uptake. Neither basal nor insulin-stimulated glucose transport was altered by 100 μmol/L sodium orthovanadate, which clearly potentiated the mild stimulatory action of hydrogen peroxide. Among fifteen biogenic amines tested, adrenaline and noradrenaline were the most efficient in activating 2-deoxyglucose uptake. The stimulation occurred within 0.01-1 mmol/L dose range and was not enhanced with vanadium. Although known to be monoamine oxidase substrates, the stimulation induced by adrenaline and noradrenaline resisted the blockade by amine oxidase inhibitors, as previously found for rodent adipocytes. The tested α- and β-adrenergic agonists did not stimulate glucose uptake in human adipocytes, and the effects of catecholamines were not inhibited by adrenergic antagonists. Benzoquinone and pyrocatechol, two of the various metabolites of catecholamine catabolism were ineffective. Only catalase, together with the antioxidant glutathione, impaired the adrenaline stimulated glucose uptake.

Research conclusions

The powerful synergism of vanadium/catecholamines previously reported on rodent adipocytes was not detectable in human fat cells. Nevertheless, adrenaline and noradrenaline were more stimulatory of hexose uptake than equivalent doses of vanadate, in a manner that was independent from adrenoceptor stimulation or amine oxidase activity.

Research perspectives

If future studies demonstrate an improvement of the antidiabetic properties of vanadium complexes *via* their combination with catecholamines, such improvement will likely not be the result of a synergistic effect on the glucose handling by fat cells.

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ORIGINAL ARTICLE

Retrospective Study

Role of nutritional ketosis in the improvement of metabolic parameters following bariatric surgery

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Informed consent statement: All study participants, or their legal guardian, provided informed

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Abstract

BACKGROUND

Ketone bodies (KB) might act as potential metabolic modulators besides serving as energy substrates. Bariatric metabolic surgery (BMS) offers a unique opportunity to study nutritional ketosis, as acute postoperative caloric restriction leads to increased lipolysis and circulating free fatty acids.

AIM

To characterize the relationship between KB production, weight loss (WL) and metabolic changes following BMS.

METHODS

For this retrospective study we enrolled male and female subjects aged 18-65 years who underwent BMS at a single Institution. Data on demographics, anthropometrics, body composition, laboratory values and urinary KB were collected.

RESULTS

Thirty-nine patients had data available for analyses [74.4% women, mean age 46.5 ± 9.0 years, median body mass index 41.0 (38.5; 45.4) kg/m^2, fat mass 45.2% ±

written consent prior to study enrollment.

Conflict-of-interest statement: The authors have none to declare.

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6.2%, 23.1% had diabetes, 43.6% arterial hypertension and 74.4% liver steatosis]. At 46.0 ± 13.6 d post-surgery, subjects had lost 12.0% ± 3.6% of pre-operative weight. Sixty-nine percent developed ketonuria. Those with nutritional ketosis were significantly younger [42.9 (37.6; 50.7) years *vs* 51.9 (48.3; 59.9) years, P = 0.018], and had significantly lower fasting glucose [89.5 (82.5; 96.3) mg/dL *vs* 96.0 (91.0; 105.3) mg/dL, P = 0.025] and triglyceride levels [108.0 (84.5; 152.5) mg/dL *vs* 152.0 (124.0; 186.0) mg/dL, P = 0.045] *vs* those with ketosis. At 6 mo, percent WL was greater in those with postoperative ketosis (-27.5% ± 5.1% *vs* 23.8% ± 4.3%, P = 0.035). Urinary KBs correlated with percent WL at 6 and 12 mo. Other metabolic changes were similar.

CONCLUSION
Our data support the hypothesis that subjects with worse metabolic status have reduced ketogenic capacity and, thereby, exhibit a lower WL following BMS.

Key Words: Obesity; Ketone bodies; Bariatric surgery; Weight loss; Glucose metabolism; Lipid metabolism

Core Tip: Ketone bodies might act as potential metabolic modulators besides serving as energy substrates. Acute postoperative caloric and carbohydrate restriction after bariatric metabolic surgery (BMS) leads to increased lipolysis, inducing ketogenesis. We report that the majority, but not all patients undergoing BMS, develop nutritional ketosis. Patients with nutritional ketosis had significantly lower baseline fasting glucose and triglyceride levels *vs* those without ketonuria. Weight loss was greater in those with postoperative ketonuria, and urinary ketones positively correlated with percent weight loss. These observations suggest that subjects with worse gluco-metabolic status have reduced ketogenic capacity, which might blunt the metabolic response to BMS.

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INTRODUCTION

Ketogenesis primarily occurs in the liver at rates proportional to total fat oxidation under conditions of reduced glucose availability such as fasting or very low-calorie ketogenic diets (VLCKDs). In brief, under these conditions, lipolysis-derived free fatty acids (FFAs) undergo beta-oxidation and are broken down into acetyl CoA, which is then converted to ketone bodies (KB), namely acetone, acetoacetate (AcAc), and beta-hydroxy butyrate (BHB), in the mitochondrial matrix of hepatocytes. KBs, namely BHB, AcAc and acetone, transfer lipid-derived energy from the liver, which cannot use them as a fuel, to extrahepatic organs (*e.g.*, central nervous system, heart, skeletal muscle, kidney), serving as an energy substrate alternative to glucose[1]. Over the past few years, the interest in KBs and nutritional ketosis has progressively increased, largely due to the discovery that, besides serving as energy substrates, KBs may also exert favourable metabolic effects[2,3], serving as metabolic regulators and signalling molecules. In particular, BHB exerts antioxidant and anti-inflammatory effects, may affect epigenetics by inhibiting histone deacetylation, suppresses the activity of the sympathetic nervous system and reduces lipolysis and, through unknown mechanisms, to play a role in appetite suppression[4,5]. In healthy individuals, even small increases in KB levels were shown to lower glucose and circulating FFA independent of insulin and glucagon[6], and to attenuate the glycaemic response to an oral glucose tolerance test by increasing insulin sensitivity[7], suggesting a direct metabolic effect of KBs. Bariatric metabolic surgery (BMS) offers a unique opportunity to study

nutritional ketosis, avoiding the complexity of a nutritional intervention such as VLCKD that would need greater effort from patients and also greater costs[8]. Similar to VLCKDs, BMS involves a marked energy deficit that results in massive mobilization of FFAs from adipose tissue and therefore ketogenesis[9,10]. The role of BMS in achieving sustained weight loss (WL), improving obesity-related comorbidities and reducing mortality is well established[11]. However, not all subjects respond to a similar extent[12], those with cardiometabolic abnormalities such as diabetes (especially when long-standing or poorly controlled) and arterial hypertension exhibiting poorer WL after surgery[13,14]. To the best of our knowledge, no studies have assessed the relationship between ketogenic capacity, as reflected by KB production in response to marked calorie restriction, and WL after BMS. We hypothesized that subjects with reduced ketogenic capacity are poorer responders to BMS in terms of WL 6 mo surgery.

MATERIALS AND METHODS

Study design

This was an observational, retrospective, single-centre study part of the KETO-BMS study. Male and female subjects aged 18-65 years who underwent laparoscopic sleeve gastrectomy at San Raffaele Scientific Institute from May 2016 to November 2018 and had urinary KB measured within two months of surgery and a follow-up of at least 6 mo were included. The protocol was approved by the Institutional Ethics Committee, and all patients provided informed consent. All patients underwent routine assessments prior to BMS, including medical history, physical examination, measurement of anthropometrics [height (cm), weight (kg) and body mass index (BMI), calculated as the ratio between the weight and the height squared, waist circumference (WC)], body composition (measured by electric bio-impedance in the fasting state using a BIA AKERN device and the software Bodygram PLUS software, Akern, Montachiello, Italy). Metabolic parameters including fasting plasma glucose (FPG), total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides were collected. During the first 8 wk after surgery, patients meet with a registered dietitian and subsequently with a staff physician for nutritional assessment and guidance. As per institutional protocols, during this time frame patients move from clear liquids to pureed foods, progressively increasing to approximately 750-900 kcal daily, depending on protein requirements (up to 1.5g/kg IBW). After the first 8 wk, patients move to solid foods and gradually increase the daily energy intake. Assessments are scheduled every 3-6 mo for the first 12 mo, and annually thereafter. Follow-up outpatient visits include medical history review, physical examination, measurement of anthropometrics, and laboratory assessments as per current recommendations[15].

KB production

KB production was assessed by the presence of acetoacetic acid in urine using an automated dipstick urinalysis (Aution MAX and Aution Sticks, Menarini Diagnostics, Florence, Italy). This is a semiquantitative method that detects urinary acetoacetic acid at concentrations ranging from 5 mg/dL to 150 mg/dL.

Statistical analysis

Descriptive statistics were obtained for all study variables. Normality was assessed with the Shapiro-Wilk test. Continuous variables were expressed as mean ± SD or median (25^{th}-75^{th} percentile), depending on data distribution. Categorical variables were summarised as counts and percentages. Missing data were not imputed. The *t*-test, Welch *t*-test, or Mann-Whitney *U*-test were used for between-group comparisons, depending on variable distribution. The Fisher's exact test was used to assess the association between categorical variables and KB production.

Our primary objective was to examine the relationship between KB production and WL at 6 mo after BMS. One-way analyses of covariance were conducted to examine the effect of sex and pre-operative cardiometabolic conditions [diabetes mellitus (DM), hypertension, dyslipidaemia] on WL at 6 mo, with age included as a covariate. Bivariate correlation analyses were performed to examine the relationship of WL at 6 mo with pre-operative BMI, fat mass, FPG, total cholesterol, triglycerides and post-operative urinary KBs. Relevant variables that were significantly correlated were included in a hierarchical multiple-regression analysis, while controlling for sex, age and BMI. All variables were screened for violations of the assumptions relevant to

each of the statistical analysis performed. Statistical significance was set at $P < 0.05$. Statistical analysis was conducted using IBM SPSS Statistics (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, United States: IBM Corp.).

RESULTS

Patient population

A total of 39 patients were included in the analysis. Patient characteristics are depicted in Table 1. Patients were middled-aged, mostly females. Metabolic-associated fatty liver disease was the most prevalent obesity complication, followed by dyslipidaemia, hypertension and DM.

KB production

Most patients (69.2%) developed ketosis after a mean of 46.0 ± 13.6 d from surgery. Time from surgery was similar between those who did or did not develop ketosis (44.6 ± 15.0 d *vs* 49.0 ± 9.5 d, respectively; $P = 0.351$). Patients with ketosis were significantly younger and had significantly lower pre-operative FPG and triglyceride levels, but greater LDL cholesterol (Table 2). Urinary KBs were inversely correlated with age (Spearman's rho -0.519, $P = 0.001$), FPG (Spearman's rho -0.366, $P = 0.024$), and positively correlated with LDL cholesterol (Spearman's rho 0.426, $P = 0.011$). There was no correlation between urinary KBs and BMI ($P = 0.936$), WC ($P = 0.619$), percent fat mass ($P = 0.768$), total cholesterol ($P = 0.368$), HDL cholesterol ($P = 0.618$) or triglycerides ($P = 0.095$).

WL after surgery

Mean WL at 6 mo was 26.4% ± 5.1% of pre-operative weight in the whole group. WL at 6 mo was significantly greater in patients who had developed post-operative ketosis ($P = 0.035$; Figure 1). Time of assessment was similar between those who did or did not develop ketosis (6.1 ± 0.9 mo *vs* 6.1 ± 0.5 mo, respectively; $P = 0.931$). In 35 patients who had available data at 12 mo (89.7% of the total, 24 and 11 in the group with and without ketosis, respectively), WL also tended to be greater in those with post-operative ketosis ($P = 0.067$, Figure 1). Time of assessment was similar between those who did or did not develop ketosis (11.9 ± 0.9 mo *vs* 12.1 ± 1.2 mo, respectively; $P = 0.590$).

Urinary KB (Spearman's rho 0.398, $P = 0.012$) significantly correlated with WL at 6 mo, whereas age ($P = 0.290$), BMI ($P = 0.056$), fat mass ($P = 0.735$), FPG ($P = 0.680$), total cholesterol ($P = 0.508$) and triglycerides ($P = 0.976$) did not. After adjustment for age, there was a statistically significant difference in WL at 6 mo between males and females, $F(1, 36) = 5.221$, $P = 0.028$, partial $\eta^2 = 0.127$. There was no statistically significant difference between patients with or without DM, hypertension, or dyslipidaemia, therefore these variables were not included in the regression model. At hierarchical multiple regression, urinary KBs and male sex emerged as significant predictors of WL at six months. The full model statistically significantly predicted WL at 6 mo, $R^2 = 0.31$, $F(4, 34) = 3.76$, $P = 0.012$ (Table 3). Urinary KBs also correlated with WL at 12 mo (Spearman's rho 0.356, $P = 0.036$).

Laboratory variables at 6 mo were available for a subgroup of patients. No statistically significant differences in percent change from pre-surgery to 6 mo were detected between groups (Figure 2), although patients who had developed nutritional ketosis tended to have a greater percent increase in HDL cholesterol and greater percent reductions in total and LDL-cholesterol, whereas those who did not develop ketosis tended to have a greater reduction in triglycerides.

DISCUSSION

In this analysis of patients who underwent BMS, we found that KB production during marked calorie restriction after surgery predicted WL at 6 mo. Patients who developed nutritional ketosis had greater WL at 6 mo and tended to have a greater WL at 12 mo after surgery, as compared with those who did not develop nutritional ketosis.

Little information is available on KB production after BMS. Crujeiras *et al*[9] reported that patients who underwent BMS developed mild ketosis at one month after surgery. Thereafter, KBs decreased and returned to pre-operative levels at 3 mo. The association between nutritional ketosis and WL was not explored in that study, which

Table 1 Pre-operative patient characteristics

	All 39	Missing
Age, yr	46.5 ± 9.0	-
Male, *n* (%)	10 (25.6)	-
Hypertension, *n* (%)	17 (43.6)	-
Diabetes mellitus, *n* (%)	9 (23.1)	-
Dyslipidaemia, *n* (%)	22 (56.4)	-
MAFLD	29 (74.4)	-
Waist circumference (cm)		2
Males	129.7 ± 6.2	
Females	114.1 ± 13.3	
BMI, kg/m^2	41.0 (38.5; 45.4)	-
Fat mass (%)	45.2 ± 6.2	5
Plasma glucose (mg/dL)	91.0 (84.0; 98.3)	1
Total cholesterol (mg/dL)	193.1 ± 29.6	3
HDL cholesterol (mg/dL)	48.0 (42.0; 58.0)	4
LDL cholesterol (mg/dL)	115.1 ± 28.0	4
Triglycerides (mg/dL)	118.5 (102.3; 159.3)	3

MAFLD: Metabolic-associated fatty liver disease; BMI: Body mass index; HbA1c: Glycated haemoglobin; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

had a different aim. There may be different explanations for the association between post-operative nutritional ketosis and the greater WL at 6 mo observed in our study. It has been reported that conditions of altered glucose metabolism such as type 2 DM negatively impact WL after BMS[16,17]. Ketogenic capacity might be a proxy of glucometabolic health. Previous studies suggested that ketogenic capacity is impaired in women with obesity as compared to normal-weight controls[18], in the pathogenesis of non-alcoholic liver disease and progression to non-alcoholic steatohepatitis, and even hepatocellular carcinoma[19-21]. Furthermore, studies in mice indicate that impaired ketogenesis may play a role in fatty liver injury and dysregulated glucose homeostasis[22-24]. Patients who did not develop nutritional ketosis in our cohort had significantly higher FPG and triglycerides, indicating worse glucometabolic status. Impaired ketogenesis may be responsible for a diminished extraction of available fat, altered acetyl-CoA balance in mitochondria, and diversion of non-disposed FFAs to other metabolic pathways, possibly including lipogenesis [23]. Conversely, better WL and metabolic responses to BMS in patients with adequate ketogenic capacity might be due to efficient clearance of excess FFAs released from adipose tissue. It has been known for more than 40 years that KBs may have roles beyond serving as energy substrates[25]. Specifically, BHB appears to exert antioxidant and anti-inflammatory effects, to inhibit histone deacetylation and to play a role in appetite suppression[4,5]. In healthy individuals, even small increases in circulating KBs were shown to reduce glucose and triglyceride levels, and to hamper the glycaemic response to an oral glucose load by increasing insulin sensitivity[6,7]. At the time of KB assessment, WL was similar between patients with or without ketosis. However, it is tempting to speculate that exposure to mild ketosis led to an improvement of mitochondrial bioenergetics and metabolic health[3,26,27], which in turn resulted in improved subsequent WL. Despite having significantly higher LDL cholesterol prior to surgery, patients who developed nutritional ketosis exhibited a numerically greater reduction in LDL at 6 mo as compared with patients who did not develop nutritional ketosis (Figure 2). During ketogenesis, acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by mitochondrial HMG-CoA synthase, an enzyme that is also involved in cholesterol synthesis[28]. It is possible that, in conditions of low glucose and high FFA availability, an increase in ketogenesis results in lower rates of *de novo* cholesterol synthesis.

Table 2 Comparison of pre-operative characteristics between subjects who developed (patients with post-operative ketosis) or did not develop (patients without post-operative ketosis) ketosis after surgery

	KB+ (*n* = 27)	KB- (*n* = 12)	*P* value
Age, yr	42.9 (37.6; 50.7)	51.9 (48.3; 59.9)	**0.018**
Female, *n* (%)	20 (74.1)	9 (75.0)	1.000
Hypertension, *n* (%)	11 (40.7)	6 (50.0)	0.730
Diabetes mellitus, *n* (%)	4 (14.8)	5 (41.7)	0.102
Dyslipidaemia, *n* (%)	14 (51.9)	8 (66.7)	0.494
MAFLD	20 (74.1)	9 (75.0)	0.683
Waist circumference[1] (cm)	119.3 ± 13.5	115.3 ± 14.0	0.421
BMI, kg/m^2	41.0 (38.7; 45.4)	40.1 (35.9; 45.6)	0.663
Fat mass (%)	45.6 ± 6.2	44.2 ± 6.1	0.552
Plasma glucose (mg/dL)	89.5 (82.5; 96.3)	96.0 (91.0; 105.3)	**0.025**
HbA1c (mmol/mol)	37.0 (35.8; 41.0)	38.5 (36.0; 46.3)	0.305
Total cholesterol (mg/dL)	197.1 ± 25.8	183.9 ± 36.4	0.222
HDL cholesterol (mg/dL)	48.0 (42.5; 53.0)	49.0 (39.5; 62.0)	0.843
LDL cholesterol (mg/dL)	121.0 ± 23.5	100.2 ± 33.9	**0.045**
Triglycerides (mg/dL)	108.0 (84.5; 152.5)	152.0 (124.0; 186.0)	**0.020**

[1]Pooled data for males and females, as there were only 2 males in the patients without post-operative ketosis group.
MAFLD: Metabolic-associated fatty liver disease; BMI: Body mass index; HbA1c: Glycated haemoglobin; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; KB: Ketone bodies; WL: Weight loss.

Table 3 Hierarchical regression analysis for weight loss at 6 mo

	Weight loss at 6 mo					
	Model 1		Model 2		Model 3	
Variable	B	β	B	β	B	β
Constant	30.300[b]		22.386[b]		16.984	
Age	-0.106	-0.186	-0.113	-0.199	-0.032	-0.057
Sex (male)	4.038[a]	0.305	3.756[a]	0.325	4.391[a]	0.380
BMI			0.200	0.201	0.203	0.204
Urinary KB					0.074[a]	0.365
R^2	0.157		0.196		0.307	
F	3.351[a]		2.852		3.759[a]	
ΔR^2	0.157		0.040		0.110	
ΔF	3.351[a]		1.722		5.402[a]	

[a]$P < 0.05$.
[b]$P < 0.01$.
BMI: Body mass index; KB: Ketone bodies.

Differences in KB production might also be due to differences in diet macronutrient composition. A limitation of our study is that we did not record food intake in the first weeks following BMS. However, all patients received standard dietary recommendations, and compliance was reviewed by dieticians at follow-up assessments. Ketosis develops in conditions of reduced glucose availability and marked calorie restriction [29], such as in the first weeks after BMS. Following BMS, protein-rich foods are prioritized over other foods in order to prevent excess loss of fat-free mass[30]. It is

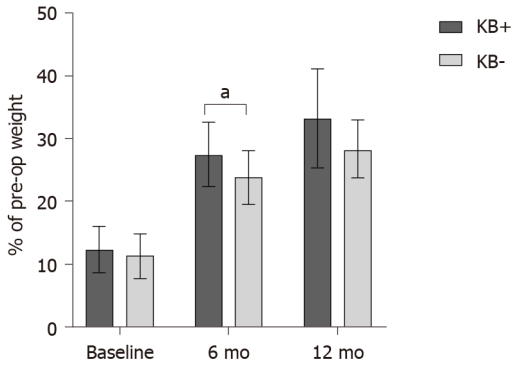

Figure 1 Weight loss at baseline (46.0 ± 13.6 d post-surgery), 6 mo and 12 mo after surgery. KB+: Patients with post-operative ketosis; KB-: Patients without post-operative ketosis. [a]*P* < 0.05.

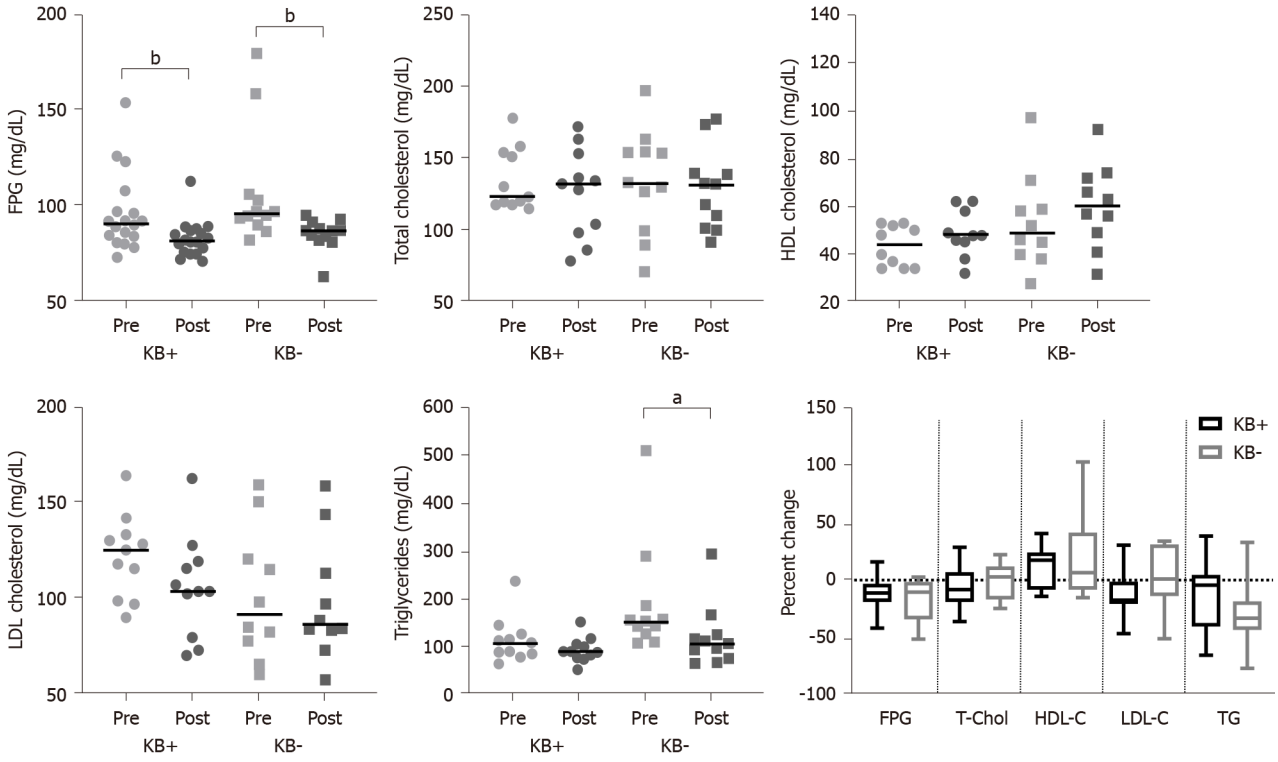

Figure 2 Changes in metabolic parameters at 6 mo after surgery. FPG: Fasting plasma glucose; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; KB+: Patients with post-operative ketosis; KB-: Patients without post-operative ketosis. [a]*P* < 0.05. [b]*P* < 0.01.

unlikely that some patients ingested relatively high amounts of carbohydrates in the first postoperative weeks. On the other hand, it is possible that some greatly restricted carbohydrates to allow adequate protein intake. Deriving energy from proteins is an expensive process for the body, which may lead to calorie consumption and greater WL as compared with diets that rely on carbohydrates as the main energy source[31-33]. In fact, during carbohydrate restriction most of the body's glucose requirements are satisfied by gluconeogenesis from amino acids, a process that requires approximately 400-600 kcal/d[32]. In other settings, several studies have demonstrated that very-low carbohydrate ketogenic diets are associated with greater WL as compared to other dietary regimens[34-36]. Diet composition in the first postoperative weeks might influence subsequent WL even in patients undergoing BMS. Other potential limitations are the relatively small sample size and the availability of data on WL at 12 mo only for a subgroup of patients, which might explain the lack of a statistically significant between-group difference in WL at this timepoint. Finally, we did not formally assess the level of physical activity throughout the 12-month follow-up to detect differences that might influence WL. In general, changes in physical activity during the first 6 mo after BMS (*i.e.*, the timepoint for the assessment of the primary

outcome in this study) are small and unlikely to affect WL[37]. We cannot exclude that changes in physical activity during the following months influenced WL at 12 mo.

CONCLUSION

In conclusion, it is possible that both metabolic status and diet composition influenced KB production in our cohort. Urinary KBs are easy to measure, and could be an early predictor of WL after BMS. Increasing evidence indicates that nutritional ketosis may have several health benefits[2,22,38-49]. Our findings add to this knowledge, suggesting that patients who develop nutritional ketosis following BMS might have greater WL and better metabolic responses to BMS.

ARTICLE HIGHLIGHTS

Research background

Ketone bodies (KB) derived from free fatty acid (FFA) metabolism serve as energy substrates in conditions of reduced glucose availability, but also as metabolic regulators and signalling molecules. Bariatric metabolic surgery (BMS) involves a marked energy deficit that results in massive mobilization of FFAs from adipose tissue, resulting in the activation of ketogenesis. It is not known whether all subjects undergoing BMS become ketotic, and whether there is a relationship between ketogenic capacity and weight loss (WL) following BMS.

Research motivation

We hypothesized that subjects with reduced ketogenic capacity are poorer responders to BMS in terms of WL. Characterization of the relationship between ketogenic capacity and WL following BMS will help understand the metabolic actions of KB and find out whether KB could be used as a predictor of BMS-induced WL.

Research objectives

We assessed the relationship between KB production in the first weeks after BMS and WL at 6 mo. We also assessed the relationship of KB with metabolic parameters and WL at 12 mo.

Research methods

For this retrospective study, we analyzed data from 39 patients who underwent laparoscopic sleeve gastrectomy, had urinary KB measured within two months of surgery and a follow-up of at least 6 mo. KB production was assessed by the presence of acetoacetic acid in urine using an automated dipstick urinalysis. We compared patients who developed post-operative ketosis with those who did not. The relationship of WL at 6 mo with pre-operative anthropometrics, body composition and metabolic parameters, and with post-operative urinary KBs was studied using bivariate correlation analyses. Variables that were significantly correlated were included in a hierarchical multiple-regression analysis, while controlling for sex, age and BMI.

Research results

This was the first study to specifically assess the relationship of ketogenic capacity with weight and metabolic outcomes. Most, but not all patients (69.2%), developed ketosis after a mean of 46.0 ± 13.6 d from surgery. Patients with ketosis were significantly younger [42.9 (37.6; 50.7) years *vs* 51.9 (48.3; 59.9) years, $P = 0.018$] and had significantly lower pre-operative fasting plasma glucose [89.5 (82.5; 96.3) mg/dL *vs* 96.0 (91.0; 105.3) mg/dL, $P = 0.025$] and triglyceride levels [108.0 (84.5; 152.5) mg/dL *vs* 152.0 (124.0; 186.0) mg/dL, $P = 0.020$], but greater LDL cholesterol (121.0 ± 23.5 mg/dL *vs* 100.2 ± 33.9 mg/dL, $P = 0.045$). WL at 6 mo was significantly greater in patients who had developed post-operative ketosis (27.5% ± 5.1% *vs* 23.8% ± 4.3% in the groups with and without ketosis, respectively; $P = 0.035$). At hierarchical multiple regression, urinary KBs and male sex emerged as significant predictors of WL at 6 mo.

Research conclusions

In keeping with the growing body of evidence indicating that nutritional ketosis has

several health benefits, our findings suggest that patients who develop nutritional ketosis following BMS might have greater WL and better metabolic responses to BMS.

Research perspectives

Our findings should be considered hypothesis-generating. Further research is needed to confirm these data in larger populations, and to assess the relationship between ketogenic capacity and metabolic responses to BMS with more sophisticated techniques.

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LETTER TO THE EDITOR

Gut microbiota-derived metabolites are novel targets for improving insulin resistance

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Abstract

The gut microbiota plays a key role in metabolic diseases. Gut-microbiota-derived metabolites are found in different dietary sources, including: Carbohydrate (acetate, propionate, butyrate, also known as short-chain fatty acids, as well as succinate); protein (hydrogen sulfide, indole, and phenylacetic acid); and lipids (resveratrol-, ferulic acid-, linoleic acid-, catechin- and berry-derived metabolites). Insulin resistance, which is a global pandemic metabolic disease that progresses to type 2 diabetes mellitus, can be directly targeted by these metabolites. Gut-microbiota-derived metabolites have broad effects locally and in distinct organs, in particular skeletal muscle, adipose tissue, and liver. These metabolites can modulate glucose metabolism, including the increase in glucose uptake and lipid oxidation in skeletal muscle, and decrease in lipogenesis and gluconeogenesis associated with lipid oxidation in the liver through activation of phosphatidylinositol 3-kinase - serine/threonine-protein kinase B and AMP-activated protein kinase. In adipose tissue, gut-microbiota-derived metabolites stimulate adipogenesis and thermogenesis, inhibit lipolysis, and attenuate inflammation. Importantly, an increase in energy expenditure and fat oxidation occurs in the whole body. Therefore, the therapeutic potential of current pharmacological and non-pharmacological approaches used to treat diabetes mellitus can be tested to target specific metabolites derived from intestinal bacteria, which may ultimately ameliorate the hyperglycemic burden.

Key Words: Insulin resistance; Gut microbiota; Metabolites; Host metabolism; Metabolic organs; Novel targets

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Core Tip: The gut-microbiota-derived metabolites play a key role in metabolic diseases. Insulin signaling pathways are directly targeted by these metabolites, as they promote an increase in glucose uptake and lipid oxidation in skeletal muscle; a decrease in lipogenesis and gluconeogenesis associated with an increase in lipid oxidation in the liver; and an improvement in thermogenesis and inflammation in the adipose tissue. Collectively, these findings pave the way for the development of novel drugs or for investigation of the therapeutic potential of drugs currently used to treat insulin resistance, targeting the gut-microbiota-derived metabolites.

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TO THE EDITOR

We read with interest the recent publication by Jang and Lee[1] on the relationship of mechanisms linking the gut microbiota-derived metabolites to insulin resistance published in this journal.

The gut microbiota plays a key role in metabolic diseases. Gut-microbiota-derived metabolites are found in different dietary sources, including: Carbohydrate (acetate, propionate, butyrate, and succinate); protein (hydrogen sulfide, indole, and phenylacetic acid); and lipids (resveratrol-, ferulic acid-, linoleic acid-, cathecin- and berry-derived metabolites). Insulin signaling pathways are directly targeted by these metabolites. Therefore, gut-microbiota-derived metabolites, in particular, the short-chain fatty acids (SCFAs), increase glucose uptake and lipid oxidation in skeletal muscle, whereas in the liver, SCFAs decrease lipogenesis and gluconeogenesis, increasing the lipid oxidation through activation of phosphatidylinositol 3-kinase - serine/threonine-protein kinase B (PI3K-AKT-PKB) and AMP-activated protein kinase. In adipose tissue, SCFAs stimulate adipogenesis and thermogenesis, inhibit lipolysis, and attenuate inflammation. Therefore, an increase in energy expenditure and fat oxidation occurs in the whole body. Collectively, these findings pave the way for the development of novel drugs or for investigation of the therapeutic potential of drugs currently used to treat insulin resistance, targeting the gut-microbiota-derived metabolites.

Notably, preclinical models and clinical studies substantiate the interaction between intestinal microbiota and the pathophysiology of insulin resistance in type 2 diabetes mellitus (DM)[2-4].

Therefore, this current article provides an overview of the important role of the specific microbiota-derived compounds in insulin-responsive tissues, acting as risk factors or protectors for the development of insulin resistance, and highlights the biologic implications of the muscle–liver–adipose tissue axis interaction.

Even though the authors documented the potential role of some bacterial metabolites as regulators of metabolic functions in the body, such as SCFAs derived from carbohydrates (propionate, butyrate and acetate), and the protein- and lipid-derived metabolites, in modulating pathways of insulin signaling, the impact of these bacterial metabolites on host metabolism warrants further investigation.

Importantly, succinate is a metabolite of the tricarboxylic acid cycle and is produced equally by microbiota and the host[5]. Although this metabolite contributes to improving glucose homeostasis through the activation of intestinal gluconeogenesis [6], in obese individuals, high levels of this circulating metabolite are documented[5]. Furthermore, the imbalance of higher relative abundance of succinate-producing bacteria (Prevotellaceae and Veillonellaceae) and lower relative abundance of succinate-consuming bacteria Odoribacteraceae and Clostridaceae) may promote an increase in succinate levels and, ultimately, impaired glucose metabolism. These authors also pointed out succinate as having a potential role in metabolic-associated cardiovascular disorders and obesity. Additionally, succinate acts as an immunogenic molecule, identified as damage-associated molecular patterns. This molecule is recognized by immune cells and stabilizes hypoxia-inducible factor-1α through its G-protein coupled receptor (succinate receptor 1/SUCNR1 or GPR19), which leads to the

proinflammatory differentiation of T lymphocytes, and production of cytokines through interaction with Toll-like receptor ligands in dendritic cells[7,8]. Collectively, these findings may promote an enhancement of insulin resistance and DM burden.

Furthermore, hydrogen sulfide (H_2S) and the role of sulfur-reducing bacteria from the intestinal microbiota have gained insights into the physiological implications of host glycemic control[9]. Thus, H_2S metabolite may protect against oxidative stress by restoring reduced glutathione (GSH) and scavenging of mitochondrial reactive oxygen species, inducing pro-survival/angiogenesis signaling pathway (STAT3, signal transducer and activator of transcription 3), and promoting immunomodulation (inhibition/activation of nuclear factor-κB) and vasodilation (activation of K_{ATP} ion channel)[10]. However, the balance between therapeutic and harmful effects of H_2S should be considered when targeting that metabolite, as H_2S either endogenous or exogenous, as well as that produced by the gut microbiota, promotes or inhibits a variety of characteristics in mucosal microbiota biofilms[11]. Depending on H_2S concentration, in particular, when the gut microbiota produces an excessive amount, it may cause mucus disruption and inflammation in the colon and contribute to cancer. Conversely, low levels of H_2S directly stabilize mucus layers, prevent fragmentation and adherence of the microbiota biofilm to the epithelium, inhibit the release of invasive opportunistic pathogens or pathobionts, and prevent inflammation and tissue injury[11]. Moreover, H_2S overproduction is a causative factor in the pathogenesis of β-cell death in DM due to increased levels of reactive oxygen and nitrogen species, whereas its deficiency, as a result of increased H_2S consumption by hyperglycemic cells, may lead to endothelial dysfunction, and kidney and heart diseases[12].

As we learn more about gut-microbiota-derived metabolites, we will better understand how to target these metabolites. Thus, acetate, which is involved in host energy, substrate metabolism, and appetite *via* secretion of the gut hormones [glucagon-like peptide (GLP) and peptide YY], may be increased by oral acetate administration (vinegar intake), colonic acetate infusions, acetogenic fibers and acetogenic probiotic administration[13]. These strategies may both decrease whole-body lipolysis and systemic proinflammatory cytokine levels, and increase energy expenditure, insulin sensitivity, and fat oxidation, which contributes to weight control and glucose homeostasis. Probiotics (live microorganisms) act as microbiome modulators and confer a health benefit, as demonstrated by the capacity of selected probiotic strains (lactobacilli and enterococci) to increase SCFA production; in particular, propionate and butyrate[14]. As reviewed elsewhere, probiotic administration (*Bifidobacterium pseudocatenulatum*, *Lactobacillus plantarum*, or the formula VSL#3) in preclinical models of obesity led to an increase in the intestinal barrier function, a reduction in the endotoxemia, acceleration in metabolism, and suppression of body weight gain and insulin resistance *via* modulation of the gut microbiota composition and SCFA production[15]. Probiotics may also ameliorate glucose homeostasis and lipid profile in diabetic mice[15].

From a clinical point of view, obese children treated with the probiotic *Lactobacillus casei* shirota for 6 mo presented with loss of weight, improved lipid metabolism, and an increase in the number of *Bifidobacterium* spp. and acetate concentration in the feces [16]. Likewise, patients with type 2 DM treated with probiotics containing *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. lactis BB-12 for 6 wk had improved glucose and lipid profiles, which were associated with lower levels of systemic inflammation and increased concentration of acetate[17]. Additionally, modification of gut microbiota by dietary weight loss intervention decreased circulating succinate levels and improved the metabolic profile in a cohort of individuals with type 2 DM and obesity[6].

Pharmacological interventions or xenobiotics may also have effects on gut microbiota. Metformin is the most frequently administered medication to treat patients with insulin resistance and type 2 DM. This drug may alter the gut microbiota composition through an increase in the Bacteroidetes and Verrucomicrobia phyla and the mucin-degrading *Akkermansia muciniphila*, *Bacteroides*, and *Escherichia* genera, as well as in butyrate and propionate production, emphasizing maintenance of the integrity of the intestinal barrier, regulation of bile acid metabolism and improvement in glucose homeostasis[18,19]. Importantly, metformin may have these benefits in newly diagnosed DM[20].

Sodium-glucose cotransporter 2 inhibitors represent the most recently approved class of oral medications for the treatment of type 2 DM. Dapagliflozin decreased the Firmicutes-to-Bacteriodetes ratio in diabetic mice, which was correlated with improvement in vascular function[21]. In a rodent model of type 1 DM, inhibition of SGLT2 reduced the intermediate metabolite succinate and increased butyrate levels, as well as decreased norepinephrine content in the kidney[22]. Hence, the impact of

SGLT2 inhibitors on the gut microbiota is an area of active research.

Likewise, GLP-1 agonists reduced the abundance of the species of the Firmicutes phylum (Lachnospiraceae and Clostridiales) and increased the abundance of the species representing the Proteobacteria (*Burkholderiales bacterium* YL45) and Verrucomicrobia (*Akkermansia muciniphila*), as well as Firmicutes (Clostridiales and Oscillospiraceae) phyla in obese mice[23]. In particular, body weight loss was associated with increased abundance of *Akkermansia muciniphila*, a mucin-degrading SCFA-producing species, whose abundance is decreased in obesity and has a negative correlation with markers of gut permeability and inflammation. Notably, the GLP-1 agonist liraglutide can prevent weight gain by modulating gut microbiota composition in both obese and diabetic obese animals[24].

In the cardiometabolic disease setting, lipid-lowering drugs, such as statins, may also play an important role in modulating gut microbiota. *In vitro* studies have documented increased levels of SCFA production, including propionate, butyrate and acetate[25]. These drugs may increase the abundance of the *Bacteroides*, *Butyricimonas* and *Mucispirillum* genera, which is associated with a decrease in the inflammatory response, including lower levels of interleukin (IL)-1β and IL-6, and higher levels of transforming growth factor β-1 in the ileum, and improved hyperglycemia[26]. In humans, obesity is associated with a microbiota signature based on the abundance of the *Bacteroides* genus profile, displaying the lowest abundances of *Akkermansia* and *Faecalibacterium*, as well as a decrease in the butyrate production potential[27]. Importantly, statin therapy resulted in a lower prevalence of a proinflammatory microbial community type in obese individuals.

In conclusion, the gut microbiota imbalances and maladaptive responses have been implicated in the pathology of insulin resistance, DM, and obesity[28]. Host-gut microbiota interaction is suggested to play a contributory role in the therapeutic effects of antidiabetics, statins, and weight-loss-promoting drugs. Therefore, additional studies combining untargeted metabolomics and proteomics are essential to identify further microbial metabolites or proteins and to determine how they interact with the host targets in improving host metabolism.

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