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EDITORIAL

Role of sodium-glucose co-transporter-2 inhibitors in the management of heart failure in patients with diabetes mellitus

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Abstract

Heart failure (HF) is a major complication of diabetes mellitus (DM). Patients with DM have considerably higher risk for HF than non-diabetic subjects and HF is also more severe in the former. Given the rising prevalence of DM, the management of HF in diabetic patients has become the focus of increased attention. In this context, the findings of several randomized, placebo-controlled trials that evaluated the effects of sodium-glucose co-transporter-2 inhibitors on the risk of hospitalization for HF in patients with type 2 DM represent a paradigm shift in the management of HF. These agents consistently reduced the risk of hospitalization for HF both in patients with and in those without HF. These benefits appear to be partly independent from glucose-lowering and have also been reported in patients without DM. However, there are more limited data regarding the benefit of sodium-glucose co-transporter-2 inhibitors in patients with HF and preserved left ventricular ejection fraction, which is the commonest type of HF in diabetic patients.

Key words: Heart failure; Type 2 diabetes mellitus; Sodium-glucose co-transporter-2 inhibitors; Canagliflozin; Dapagliflozin; Empagliflozin

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Core tip: Sodium-glucose co-transporter-2 inhibitors substantially reduce the risk of hospitalization for heart failure in patients with type 2 diabetes mellitus (T2DM). Accordingly, these agents should be considered in all patients with T2DM and HF with reduced left ventricular ejection fraction regardless of HbA_{1c} levels. However, more studies are needed to clarify the role of sodium-glucose co-transporter-2 inhibitors in patients with T2DM and HF with preserved left ventricular ejection fraction, which is the commonest type of HF in this population.

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EDITORIAL

During the last decades, the prevalence of diabetes mellitus (DM) worldwide has almost doubled, from 4.7% in 1984 to 9.3% in 2019[1]. Moreover, it is estimated that patients with DM will reach 300 million by 2025 and 366 million in 2030, with the majority of them living in low-income countries[2,3]. It has also been projected that the prevalence of DM globally will rise to 10.4% by 2040 and that 12% of healthcare expenditure will be dedicated to diabetic patients[4]. These trends are of great importance given the strong relationship between DM and cardiovascular disease (CVD). It is well-established that DM is a major cardiovascular risk factor[5]. Indeed, 75%-80% of patients with DM die due to CVD[6]. Accordingly, DM is one of the leading causes of death worldwide[7].

Among the manifestations of CVD in patients with DM, heart failure (HF) has become the focus of intense research in the last years. Heart failure is an important public health issue, affecting more than 23 million people all over the world and leading to excess morbidity and mortality[8,9]. Heart failure-related healthcare costs are also substantial and are mostly due to the repeated hospitalization of these patients[8,9]. Based on the left ventricular ejection fraction (LVEF), HF is categorized into HF with reduced EF (HFrEF), HF with midrange EF (HFmrEF) and HF with preserved EF (HFpEF)[10,11]. Patients with HFpEF have a higher prevalence of comorbidities including obesity, chronic obstructive pulmonary disease and DM than those with HFrEF[12,13]. Several studies showed that the incidence of HF is 2-5 times higher in diabetic patients than in those without DM[14,15]. Patients with type 1 DM also have a higher risk of developing HF[16]. In addition, diabetic patients with HF have longer HF-related hospital stays, more frequent HF-related readmissions and higher risk for cardiovascular mortality than patients with HF but without DM[17-20]. All-cause mortality and healthcare costs are also higher in the former[21-23].

In addition to atherosclerosis-related ischemic heart disease, small vessel dysfunction, renal dysfunction and a direct effect of insulin resistance on cardiomyocytes appear to play a role in the pathogenesis of HF in patients with DM[24,25]. The most profound feature of diabetic cardiomyopathy is LV diastolic impairment manifesting as HFpE whereas HFrEF is less prevalent in these patients[26,27]. Early signs of diastolic dysfunction in patients with DM include elevated LV filling pressures portrayed by reduced peak myocardial systolic velocity and reduced E/A ratio (transmittal early to late diastolic peak ratio), along with increased LV mass and wall thickness[28-31].

Given the rising prevalence of DM and its strong association with HF, the findings of several recent, randomized, placebo-controlled trials of sodium glucose co-transporter 2 (SGLT2) inhibitors might represent a paradigm shift in the management of these patients. In the Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients trial [n = 7020 patients with type 2 DM (T2DM) and established CVD], treatment with empagliflozin reduced the risk of hospitalization for HF by 35% and reduced the incidence of the primary composite outcome (death from cardiovascular causes, nonfatal myocardial infarction or nonfatal stroke) by 14% during a median follow-up of 3.1 years[32]. In the Canagliflozin Cardiovascular Assessment Study (n = 10142 patients with T2DM who were either ≥ 30 years old with established CVD or ≥ 50 year-old with ≥ 2 of the following cardiovascular risk factors: T2DM duration ≥ 10 years, systolic blood pressure > 140 mmHg despite treatment with ≥ 1 antihypertensive agent, current smoking, micro- or macroalbuminuria, or high-density lipoprotein cholesterol level < 39 mg/dL), treatment with canagliflozin reduced the risk of hospitalization for HF by 33% and reduced the incidence of the primary composite outcome (death from cardiovascular causes, nonfatal myocardial infarction or nonfatal stroke) by 14% during a mean follow-up of 3.6 years[33]. In the Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation trial [n = 4401 patients with T2DM and chronic kidney disease (estimated glomerular filtration rate 30-90 mL/min/1.73 m^2 and urinary albumin-to-creatinine ratio > 300 mg/g)], treatment with canagliflozin reduced the risk for hospitalization

for HF by 39% during a mean follow-up of 2.6 years[34]. In the Dapagliflozin Effect on Cardiovascular Events trial (DECLARE TIMI-58) trial (n = 17160 patients with T2DM and either established CVD or multiple cardiovascular risk factors), dapagliflozin reduced the risk for hospitalization for HF by 27% compared with placebo during a median follow-up of 4.2 years[35]. In an observational study in 309056 patients with DM followed-up in real-world practice, treatment with SGLT2 inhibitors also resulted in a 39% reduction in the risk of hospitalization for HF compared with other antidiabetic agents[36]. Notably, SGLT2 inhibitors appeared to reduce the risk of hospitalization for HF to a similar degree in patients with and without a history of HF[37,38]. It is therefore possible that SGLT2 inhibitors might prevent the development of HF in diabetic patients. However, it is also possible that many patients in these trials had undiagnosed HF and that SGLT2 inhibitors are also effective in patients with less severe, asymptomatic HF. It is also noteworthy that, in the DECLARE TIMI-58 trial, dapagliflozin reduced the risk of hospitalization for HF to a similar degree in patients with HFrEF and in those with HFpEF[38]. However, this analysis was based on a small number of patients and should be considered exploratory and hypothesis-generating[38].

Despite the consistently beneficial effects of SGLT2 inhibitors on the incidence of hospitalization for HF, it should be emphasized that only a small proportion of patients in these trials had HF at baseline (10%-15%)[32-35]. However, in the Dapagliflozin and Prevention of Adverse Outcomes in Heart Failure (DAPA-HF) trial, dapagliflozin reduced the risk of hospitalization for HF by 30% and reduced cardiovascular mortality by 18% compared with placebo in 4744 patients with New York Heart Association class II, III, or IV heart failure and an EF ≤ 40% during a median follow-up of 18.2 mo[39]. Therefore, the findings of this large study further support the benefits of SGLT2 inhibitors in the management of HF, particularly with reduced EF. Nevertheless, given the limited data on the effects of these agents in patients with HFpEF, more studies are needed in this important subgroup. It should also be mentioned that patients with DM (42% of the study population) experienced a similar reduction in the risk of hospitalization for HF as patients without DM[39]. This finding suggests that other actions of SGLT2 inhibitors besides glucose-lowering might play a role in the beneficial effects of these agents in patients with HF. Indeed, it has been reported that SGLT2 inhibitors promote reverse cardiac remodeling, improve myocardial energetics and filling conditions, reduce LV wall stress and mass and reduce blood pressure and arterial stiffness[40-43].

CONCLUSION

SGLT2 inhibitors substantially reduce the risk of hospitalization for HF in patients with DM. Accordingly, current guidelines recommend these agents in patients with T2DM and HFrEF regardless of HbA_{1c} levels[44]. However, more studies are needed to clarify the role of SGLT2 inhibitors in patients with T2DM and HFpEF, which is the most common type of HF in this population.

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REVIEW

Shared (epi)genomic background connecting neurodegenerative diseases and type 2 diabetes

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Abstract

The progressive aging of populations has resulted in an increased prevalence of chronic pathologies, especially of metabolic, neurodegenerative and movement disorders. In particular, type 2 diabetes (T2D), Alzheimer's disease (AD) and Parkinson's disease (PD) are among the most prevalent age-related, multifactorial pathologies that deserve particular attention, given their dramatic impact on patient quality of life, their economic and social burden as well the etiopathogenetic mechanisms, which may overlap in some cases. Indeed, the existence of common triggering factors reflects the contribution of mutual genetic, epigenetic and environmental features in the etiopathogenetic mechanisms underlying T2D and AD/PD. On this subject, this review will summarize the shared (epi)genomic features that characterize these complex pathologies. In particular, genetic variants and gene expression profiles associated with T2D and AD/PD will be discussed as possible contributors to determine the susceptibility and progression to these disorders. Moreover, potential shared epigenetic modifications and factors among T2D, AD and PD will also be illustrated. Overall, this review shows that findings from genomic studies still deserves further research to evaluate and identify genetic factors that directly contribute to the shared etiopathogenesis. Moreover, a common epigenetic background still needs to be investigated and characterized. The evidences discussed in this review underline the importance of integrating large-scale (epi)genomic data with additional molecular information and clinical and social background in order to finely dissect the complex etiopathogenic networks that build up the "disease interactome" characterizing T2D, AD and PD.

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Core tip: Populations' progressive aging raises important challenges to be faced, including the increased prevalence of metabolic, neurodegenerative and movement disorders, especially of type 2 diabetes, Alzheimer's disease and Parkinson's disease. These disorders are characterized by a multifactorial etiology, involving genetic and non-genetic factors, which may overlap. This review will discuss the shared (epi)genomic features, the role of mutually-associated genetic variants, common gene expression profiles and epigenetic background leading to development and progression of such disorders. Overall, this review highlights the importance of characterizing the "disease interactome" in order to establish adequate personalized and preventative healthcare approaches for the ageing populations.

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INTRODUCTION

The recent progress in medicine and the improvement of health conditions have contributed to the rise of life expectancy, on the one hand. On the other hand, the better healthcare conditions and the availability of several therapeutic approaches have run in parallel to the progressive aging of populations, which raised novel challenges to be faced by the healthcare systems and the scientific communities. In fact, the progressive aging population has resulted in the increased prevalence of chronic pathologies, especially of metabolic, neurodegenerative and movement disorders[1]. In particular, type 2 diabetes (T2D), Alzheimer's disease (AD) and Parkinson's disease (PD) are among the most prevalent chronic, age-related pathologies that deserve particular attention given their dramatic impact on patient quality of life, their economic and social burden as well the etiopathogenetic mechanisms, which may overlap in some cases.

In fact, T2D accounts for 90% of cases of diabetes mellitus, which affects 285 million people worldwide[2]. It is mainly caused by a combination of insulin resistance and relative insulin deficiency[3], which results in glucose dyshomeostasis and other concomitant conditions, including hypertension and dyslipidemia[4]. AD is characterized by progressive loss of memory and cognitive domains responsible for functional independence[5,6]. This pathology accounts for the 60%-80% of overall forms of dementia and represents the sixth cause of death in the world. It affects about 30-46 million people[5,7-9], with an increasing prevalence depending on age (ranging from 0.3%-0.5% at age 60 to 11%-15% at age 80)[10-12]. PD affects 0.3% of the worldwide population, with prevalence increasing by age; in fact, it is estimated to be 1% in people over 60 years of age and 3%-5% in individuals over 85[13,14]. The clinical features of PD include typical motor symptomatology (bradykinesia, resting tremor, postural instability, and gait difficulties) and non-motor symptoms (dysautonomia, sleep disturbances, mood, and cognitive disorders)[15,16].

T2D, AD and PD are all characterized by a multifactorial etiology, involving the interplay among genetic, epigenetic and environmental factors[17,18]. Interestingly, there are lines of evidence at the epidemiological, cognitive and neuropathological levels that seem to link T2D to AD and PD[19]. In particular, brain insulin resistance could represent the bridge linking metabolic disorders to neurodegenerative /movement pathological conditions[20]. Insulin is transported via the blood brain barrier to the central nervous system, where it regulates local blood and cerebrospinal fluid glucose levels. Nevertheless, it is thought that the principal activity in the brain may be related to the regulation of synaptic plasticity and cognitive functions[7,21]. Moreover, a little proportion of insulin may be produced in the brain, as well. Indeed,

insulin levels detected in humans and rodents have been found to be lower than those in the systemic circulation. However, differences in the levels of insulin among AD brains and age-matched controls have not been established[7]. Insulin Receptors (IRs) are well distributed in the brain, especially in the cortex, hippocampus and hypothalamus, corroborating the importance of brain insulin signaling[7,21]. The "diabetic brain" may suffer of the hyperglycemia and insulin resistance arising from the decrease in insulin receptor expression or activity[7,21]. This alteration may lead to the activation of pathogenic processes, namely enhanced production of reactive oxygen species (ROS) and pro-inflammatory cytokines, that trigger inflammatory responses also in the brain, advanced glycation products and dysfunctions of autophagic functions. Moreover, insulin resistance is able to increase the production and secretion of beta amyloid (Aβ) and alter the molecular pathways involved in the phosphorylation of Tau protein: both Aβ and hyperphosphorilated-Tau are known to misfold, aggregate and accumulate leading to the loss of synapses and death of neurons, which are typical of neurodegeneration processes[7,22-28]. Indeed, the increased neuroinflammation represents a pathological feature shared by all of the three age-related pathologies[29]. Thus, the existence of common triggering factors reflects the contribution of mutual genetic and epigenetic features in the etiopathogenetic mechanisms underlying AD, PD and T2D. On this subject, this review will summarize the shared (epi)genomic features that characterize these complex pathologies.

SHARED GENETIC MAKE-UP AND FUNCTIONAL PATHWAYS AMONG T2D, AD AND PD

Several studies have attempted to dissect the contributing genetic background(s) to determine the susceptibility to T2D, AD and PD. Concerning T2D, most of the identified genetic risk factors are mainly involved in the maintenance of β-cell homeostasis and in the modulation of insulin metabolism[2,30,31]. As previously mentioned, insulin resistance has been reported to likely influence brain functions and neuronal activity. Concerning the genetic susceptibility factors of AD and PD, several genome-wide association studies (commonly referred to as GWAS) have identified many genetic polymorphisms associated with the onset and progression of sporadic forms of AD and PD. Most of them have been found to be located within genes involved in dopamine metabolic process, apoptosis, autophagy-related pathways, Aβ cascade, Tau pathology, neuroinflammation, regulation of neuronal transmission, and survival[17,32-35]. The availability of GWAS and bioinformatic approaches has allowed for the identification of 927 single nucleotide polymorphisms (SNPs) associated with both T2D and AD in populations of European ancestry. Intriguingly, 395 of these SNPs have been reported to share the same risk allele between T2D and AD[36]. These SNPs are involved in immunity/inflammation-related pathways, cell-cell communication and neuronal plasticity, whose dysregulation may lead to increase in the neuroinflammation typically occurring in T2D and AD[7,37,38]. Polymorphisms within the *IDE/HHEX* region have also been investigated as combined susceptibility factors for T2D and AD (Table 1)[39-41]. Notably, *IDE* codes for the enzyme responsible for insulin clearance, although it is also able to degrade Aβ peptide in neurons and glia cells[7,39].

A recent study performed on populations of European ancestry has described the association of 14 common SNPs with both T2D and AD; these are located in *TP53INP1*, *NDUFAF6*, *TOMM40*, *BTBD16*, *PLEKHA1*, *PVRL2* and *APOC1* genes[42,43] (Table 1). Interestingly, these genes encode proteins involved in the regulation of autophagy, apoptosis, response to oxidative stress, mitochondrial function and lipid metabolism, and their overall dysregulation can contribute to the etiopathogenetic pathways underlying T2D and AD[7,22]. Of note, Hao *et al*[34], 2015 and Wang *et al*[39], 2017 found that both disorders shared the same risk variant in SNPs (rs10510109 and rs2421016) located in *BTBD16* and *PLEKHA1* genes (Table 1). This is of particular interest, as different SNPs within *PLEKHA1* have been associated with age-related macular degeneration (an ocular neurodegenerative complex disease)[44-46] and they map on the 10q26.13 locus, which also contains another age-related macular degeneration-associated gene (*ARMS2/HTRA1*)[47,48]. Given these data, the genetic architecture of the 10q26.13 region may be investigated for its potential contribution to neurodegeneration and could be addressed as a shared susceptibility locus for T2D and AD. Moreover, the presence of shared genetic polymorphisms associated with both diseases may also be exploited to predict the risk of developing AD in individuals already suffering from T2D.

Less information is available concerning the genetic overlap between T2D and PD. The possible link between T2D-associated genetic loci and AD/PD has been

Table 1 Subset of genetic variants and genes found to be associated with type 2 diabetes, Alzheimer's disease and Parkinson's disease, as well as those associated with type 1 diabetes and Parkinson's disease[36,39,41-43,49,50]; Biological functions have been obtained from literature data[7,22,39,49,51-56] and GeneCards (https://www.genecards.org)

Gene symbol	Gene name	Genomic location	SNP	Biological function	Potential associated diseases
IDE	Insulin degrading enzyme	10q23.33	rs6583817	Insulin clearance	T2D/AD
IDE/HHEX	Insulin degrading enzyme/hematopoietically expressed homeobox		rs1544210	Insulin clearance/transcriptional repression	
TP53INP1	Tumor protein P53 inducible nuclear protein 1	8q22.1	rs896854	Cell stress response, autophagy activation, cell cycle regulation	
TP53INP1/NDUFAF6	Tumor protein P53 inducible nuclear protein 1/NADH:Ubiquinone oxidoreductase complex assembly factor 6		rs6982393 rs4734295	Cell stress response, autophagy activation, cell cycle regulation Mitochondrial function	
NDUFAF6	NADH:Ubiquinone oxidoreductase complex assembly factor 6		rs7812465	Mitochondrial function	
TOMM40	Translocase of outer mitochondrial membrane 40	19q13.32	rs2075650		
BTBD16/PLEKHA1	BTB domain containing 16/pleckstrin homology domain containing A1	10q26.13	rs10510109	Apoptosis regulation/plasma membrane function	
PLEKHA1	Pleckstrin homology domain containing A1		rs2421016	Plasma membrane function	
PVRL2	Poliovirus receptor-like 2	19q13.32	rs6859	Cell junctions, inflammation	
APOC1	Apolipoprotein C1		rs111789331 rs12721046 rs12721051 rs4420638 rs56131196 rs66626994	Lipid metabolism	
DNM3	Dynamin 3	1q24.3	rs4504922 rs7539972	Vesicle transport, phagocytosis	
ADCY5	Adenylate cyclase 5	3q21.1	rs2877709	Chemokine signaling, insulin secretion	
CDC123	Cell division cycle 123	10p14-p13	rs11257655	Cell cycle regulation	T2D/PD
CDKN2B	Cyclin dependent kinase inhibitor 2B	9p21.3	rs2383208 rs10965250 rs10811661		
KANSL1	KAT8 regulatory NSL complex subunit 1	17q21.31	rs17661428	Transcriptional activation	T1D/PD
CXCR4	C-X-C motif chemokine receptor 4	2q22.1	rs2011946	Inflammation, neuronal development	
MAP3K14	Mitogen-activated protein kinase kinase kinase 14	17q21.31	rs2867316		
CRHR1	Corticotropin releasing hormone receptor 1		rs393152	Hormonal signaling, stress and immune response	

AD: Alzheimer's disease; PD: Parkinson's disease; T1D: Type 1 diabetes; T2D: Type 2 diabetes; SNP: Single nucleotide polymorphism.

investigated in a study involving 500 PD and 400 AD patients of Asian ancestry. The authors reported four SNPs located in *CDC123* and *CDKN2B* genes mutually associated with T2D and PD. However, this association was not confirmed after correction for multiple testing[49] (Table 1). *CDC123* and *CDKN2B* exert a role in cell

cycle regulation, and their dysfunction leads to alterations in cell homeostasis, suggesting that the genetic association with T2D and PD should be further investigated in larger cohorts and different populations. Furthermore, four different genes, namely *KANSL1*, *CXCR4*, *MAP3K14* and *CRHR1*, were found to be shared between PD and type 1 diabetes in a study aiming to evaluate the common risk factors between PD and autoimmune disorders[50] (Table 1). Intriguingly, *CXCR4* and *MAP3K14* are involved in the regulation of neuronal inflammatory responses. In particular, *CXCR4* is involved in microglia recruitment, neuronal guidance and neurodevelopmental processes[51], whereas *MAP3K14* mediates NFκB signaling (involved in immunological cytotoxicity) in brain neurons[52]. Moreover, the CXCR4 protein has been found to be overexpressed in a rodent model of diabetic neuropathic pain[53]. *KANSL1* has been found to be associated with AD, thus suggesting that the encoded protein may take part in neuronal development. Indeed, KANSL1, as part of the NLS1 complex which regulates histone acetylation, is mainly involved in the epigenetic regulation of chromatin[54]. Interestingly, mutations within *KANSL1* are able to cause intellectual disability and developmental delay[54,55]. *CRHR1* is known to be involved in the activation of hypothalamic-pituitary-adrenal axis, leading to secretion of cortisol that, in turn, causes insulin resistance. Notably, the chronic stress activated by the adrenal secretion of cortisol represents a risk factor for AD onset and progression[56]. Given these lines of evidence, the association between this set of genes and their potential involvement in etiopathogenetic pathways leading to T2D, AD and PD should be further elucidated.

In addition to the identification of shared genetic variants, the investigation of common gene expression profiles may facilitate the discovery and exploration of molecular pathways that are deregulated in T2D, AD and PD. On this subject, Rahman *et al*[57], 2018 reported intriguing insights, exploiting human gene expression datasets. Among the significant Gene Ontologies (known as GOs) and Kyoto Encyclopedia Genes and Genomes (known as KEGG) pathways shared by T2D and AD, pathways involved in glycosphingolipid biosynthesis, immune/inflammatory response, regulation of neurotransmitter transports, synaptic vesicle formation, lipid metabolism and apoptosis have been identified. T2D and PD share genes involved in immune-related networks, cell adhesion, mitochondrial activity, connective tissue/extracellular matrix organization, and synaptic maturation. Indeed, neuroinflammation may represent a common hallmark among T2D, AD and PD, given that most of the shared genes are implicated in the regulation of inflammatory networks. Interestingly, Santiago *et al*[58], 2013 found that *APP* mRNA was overexpressed in the whole blood of both T2D and PD patients. Therefore, the knowledge of shared genetic factors and gene expression profiles may help to further dissect the molecular network characterizing and linking T2D, AD and PD (Figure 1).

INSIGHTS INTO COMMON EPIGENETIC BACKGROUND(S) OF T2D, AD AND PD

The human genome is able to dynamically interact with the environment through epigenetic modifications, which altogether create the complex machinery designated to regulate lifetime and aging processes. In fact, epigenetics modulate gene expression without altering the DNA sequence. This is possible by means of different kinds of epigenetic modifications, including DNA methylation and histone modifications (which might affect gene transcription), and noncoding (nc)RNAs (which might change gene expression at the post-transcriptional level)[59]. Given the crucial role of epigenetics in the modulation of gene expression, its alteration can contribute to pathogenesis and progression of several age-related diseases, including metabolic, neurodegenerative and movement disorders[17,60]. The existence of a shared epigenetic background among T2D and neurodegenerative diseases deserves to be investigated. As a matter of fact, the gene expression signatures shared among T2D and AD/PD[57,58] may also be related to the presence of common epigenetic alterations[61]. On this subject, there are intriguing hypotheses that could be evaluated. For instance, the analysis of long-range chromatin contacts among regulatory regions and their target genes will provide insights into how epigenetic background(s) may modify chromatin conformation[62] and thus gene expression profiles in the context of T2D, AD and PD. Moreover, an interaction between micro (mi)RNA-661 and *BACE1* mRNA was found to cause a reduced expression of the resultant protein in pancreatic islets and contribute, thereby, to the development of T2D[63]. Of note, *BACE1* is involved not only in the regulation of insulin biogenesis but also in the formation of Aβ so that it could be also investigated in the etiopathogenesis of AD[64].

Furthermore, sirtuins are a family of histone deacetylases, playing critical roles in

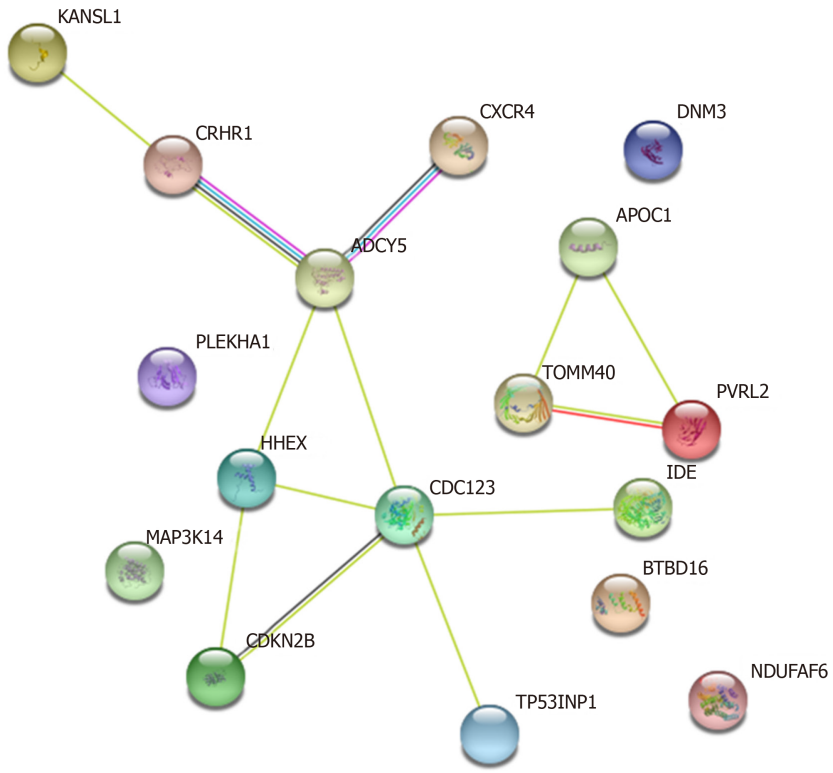

Figure 1 Known interaction networks among the potentially shared genes. Network showing the known molecular interactions (String; https://string-db.org/). The reported genes have been selected from the genetic studies discussed in the manuscript. The existence of few known molecular interactions among them highlights the need of further investigations in order to better understand the shared etiopathogenesis.

the physiology of metabolism, central nervous system, and immune system. In fact, these epigenetic modifiers are involved in a variety of molecular pathways underlying different complex diseases (cancer, diabetes, and neurodegenerative disorders)[65]. Given their role, sirtuins may be addressed as potential therapeutic targets able to counteract the progression of T2D, AD and PD through their epigenetic activity[66].

The study of DNA methylation affecting mitochondrial genes could unveil interesting insights into the pathogenesis of T2D, AD and PD. In fact, alteration of DNA methylation status has been supposed to be responsible for the reduction of complex I and IV subunits in AD and PD human brain samples[67]. Moreover, it has been demonstrated that the alteration of miR-181a/b levels impacts mitochondrial biogenesis and turnover in the brain, through the modulation of autophagy and mitophagy-related pathways[68]. These miRNAs could be, therefore, investigated for their potential role in the common pathogenetic processes leading to T2D and AD/PD. Furthermore, the study of other miRNAs and ncRNAs related to these disorders could be helpful for designing innovative class of drugs (epidrugs).

CONCLUSION

A growing body of evidence suggests the existence of multilevel networks of pathogenetic pathways which mutually contribute to the onset and progression of metabolic, neurodegenerative and movement disorders. However, few shared genetic contributors have been well characterized and a common epigenetic landscape needs to be explored. Of note, in T2D, an impairment of glucose metabolism in brain generates oxidative stress, leading to the alteration of autophagy-related pathways, mitochondrial dysfunction, increased neuronal apoptosis and, eventually, depletion of synapsis[7]. Overall, these alterations contribute to the formation of amyloid plaques and neurofibrillary tangles in AD and to the deterioration of dopaminergic neurons in different brain regions in PD[1]. As mentioned, despite a plethora of data highlighting a possible overlapping of disease mechanisms involved in T2D, AD and PD, the critical molecular and genetic features remain to be clarified. The genetic polymorphisms (Table 1) shared with T2D, AD and PD are located within genes

involved not only in brain insulin signaling but also in neuroinflammation-related pathways[34-52]. This evidence is also corroborated by the expression data obtained by the investigation of human patients and animal models presenting these pathologies[57].

Understanding the contribution of genetics, epigenetics and environment in determining the susceptibility, onset and progression of T2D, AD and PD will be crucial to achieve a deeper knowledge of metabolic, neurodegenerative and movement disorders. On this subject, the enhancement of social and cognitive activities in the high-income countries seems to strengthen the resilience against neurodegeneration, leading to a stable or reduced incidence of dementia in these regions[69,70]. On the other hand, T2D prevalence is also rising in the more developed areas[71]. Given this data and considering that T2D is regarded overall as a risk factor for dementia[72], the contribution of T2D to the development of neurodegeneration needs to be monitored. Moreover, these lines of evidence encourage the further exploration of gene-environment interactions in order to understand the similarities and the differences in the etiopathogenesis underlying AD, PD and T2D. Indeed, more comprehensive and higher resolution (epi)genomic studies should be implemented in order to collect information on genome architecture, DNA methylation, histone modifications, ncRNAs and three-dimensional genome organization. These large-scale data should be exploited to integrate genomic, epigenomic, transcriptomic, metabolomic and proteomic information with the clinical phenotype and draw the network of interactions which build up a "disease-interactome".

Indeed, the fine knowledge of the disease interactome could highlight the molecular relationships existing among T2D, AD, PD which, thereby, could be exploited to treat these conditions through a network medicine approach, able to integrate all these interactions to understand the molecular and cellular perturbations underlying diseases, providing insights and targets for the accurate diagnosis and treatment[73]. By this way, the patient could benefit from a healthcare approach based on a multilevel characterization of his condition, derived not only by clinical and molecular testing but also by his environmental and social backgrounds.

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REVIEW

Fundamentals about onset and progressive disease character of type 2 diabetes mellitus

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Abstract

ResearchGate is a world wide web for scientists and researchers to share papers, ask and answer questions, and find collaborators. As one of the more than 15 million members, the author uploads research output and reads and responds to some of the questions raised, which are related to type 2 diabetes. In that way, he noticed a serious gap of knowledge of this disease among medical professionals over recent decades. The main aim of the current study is to remedy this situation through providing a comprehensive review on recent developments in biochemistry and molecular biology, which can be helpful for the scientific understanding of the molecular nature of type 2 diabetes. To fill up the shortcomings in the curricula of medical education, and to familiarize the medical community with a new concept of the onset of type 2 diabetes, items are discussed like: Insulin resistance, glucose effectiveness, insulin sensitivity, cell membranes, membrane flexibility, unsaturation index (UI; number of carbon-carbon double bonds per 100 acyl chains of membrane phospholipids), slow-down principle, effects of temperature acclimation on phospholipid membrane composition, free fatty acids, energy transport, onset of type 2 diabetes, metformin, and exercise. Based on the reviewed data, a new model is presented with proposed steps in the development of type 2 diabetes, a disease arising as a result of a hypothetical hereditary anomaly, which causes hyperthermia in and around the mitochondria. Hyperthermia is counterbalanced by the slow-down principle, which lowers the amount of carbon-carbon double bonds of membrane phospholipid acyl chains. The accompanying reduction in the UI lowers membrane flexibility, promotes a redistribution of the lateral pressure in cell membranes, and thereby reduces the glucose transporter protein pore diameter of the transmembrane glucose transport channel of all Class I GLUT proteins. These events will set up a reduction in transmembrane glucose transport. So, a new blood glucose regulation system, effective in type 2 diabetes and its prediabetic phase, is based on variations in the acyl composition of phospholipids and operates independent of changes in insulin and glucose concentration. UI assessment is currently arising as a promising analytical technology for a membrane flexibility analysis. An increase in mitochondrial heat

production plays a pivotal role in the existence of this regulation system.

Key words: ATP; Free fatty acid; Glucose transporter; Membrane flexibility; Metformin; Slow-down principle; Type 2 diabetes; Unsaturation index

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Core tip: To maximize type 2 diabetes care the assessment of unsaturation index, as observed in erythrocytes, is strongly indicated with intervals of three months. The value of unsaturation index is a reliable parameter for controlling the acyl composition of phospholipids as modulators of membrane flexibility. Given the main role of free fatty acids in this process the assessment of free fatty acids instead of triglycerides assessment may be of benefit for monthly monitoring purposes. Counseling of exercise should be an essential element of the type 2 diabetes management plan.

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INTRODUCTION

Nine years after Banting *et al*[1] extracted insulin from a dog's pancreas, Falta *et al*[2] introduced the term "insulin resistance" in the medical literature. Based on clinical and biochemical observations, they differentiated hyperglycemia in two distinct types: A form where a relative modest dose of insulin the metabolic disorder completely compensated, referred to as "Insular Diabetes", and a form where a vast amount of insulin is well tolerated without causing a hypoglyceamia, referred to as "Insulinresistenter Diabetes". They defined the concept that insulin resistance may be one of the main causes of type 2 diabetes[2]. Five years later Himsworth[3] confirmed the principle of insulin resistance.

Since then, insulin resistance as a term describing the relative ineffectiveness of insulin was commonly used. However, MacBryde[4] wrote already in 1933: "There is as yet no general agreement as to its definition", but this remark did not come into general acceptance not even after almost 90 years. MacBryde is still a voice crying in the wilderness. A short, incomplete summary proves that MacBryde's remark is right up to the present day (Table 1)[5-12]. One of the aims of this study is to demonstrate that the recurrent phrase "insulin resistance" is an imaginary reality, *i.e.* a reality in which everyone believes, and as long as this collective belief exists this imaginary reality exerts power in the world.

GLUCOSE EFFECTIVENESS AND INSULIN SENSITIVITY

From the late 1990s, the advent of the minimal model for estimating the glucose metabolism in man has opened a new chapter to evaluate MacBryde's remark. The minimal model approach analyses the relationship between the pattern of insulin response and the rate of glucose decline to infer the sensitivity of tissues to insulin. Additionally, the model measures a relevant, less well recognized, factor called glucose effectiveness. This term describes the ability of glucose, *per se*, independent of changes in insulin concentration, to stimulate its own up-take by a mass action effect and to suppress its own release. Despite the one-compartment optimized minimal model overestimates the net glucose effectiveness, the results of this model as well as the two-compartment minimal model, which use computer modeling of glucose and insulin kinetics after intravenous glucose challenge, indicated that individuals with type 2 diabetes and individuals in the prediabetic phase had significantly lower values of both glucose effectiveness and insulin sensitivity compared to healthy controls (Table 2)[13-16]. So what this means is that insulin sensitivity (S_I) is positively related to glucose effectiveness (S_G). Moreover, a prospective study on the development of type 2 diabetes in normoglycaemic offspring of couples, who both had type 2 diabetes, showed significant defects in both S_G and S_I, *i.e.*, more than 10

Table 1 Short summary of different definitions of the term: Insulin resistance

	Ref.
Insulin resistance is the impaired sensitivity of tissue to the action of insulin	[5]
Insulin resistance signifies the inability of insulin at physiological concentrations to exert its normal metabolic actions.	[6]
Insulin resistance is a diminished ability to keep the serum glucose low with insulin levels in the normal range.	[7]
Insulin resistance is an integral concept characterizing all cases of a reduced biological effect of insulin with its normal concentration and activity.	[8]
Insulin resistance is defined as a state of reduced responsiveness to normal circulating concentrations of insulin.	[9]
Insulin resistance refers to state in which physiological concentrations of insulin are poorly effective.	[10]
Insulin resistance is characterized by a reduced sensitivity of body cells to the actions of insulin.	[11]
Insulin resistance is defined as the inability of cells to efficiently respond to stimulation by insulin.	[12]

years before the development of the disease, participants who developed the disease had lower values, compared to controls, of S_I [(3.2 ± 2.4 *vs* 8.1 ± 6.7) × 10^{-3} ·L ·min^{-1} ·$pmol^{-1}$ insulin; $P < 0.0001$] and S_G [(1.6 ± 0.9 *vs* 2.3 ± 1.2) × 10^{-2} ·min^{-1}; $P < 0.0001$][15].

To circumvent the limitations of the one-compartment minimal model, we used S_G and S_I values of stable isotope-labelled glucose data obtained from the two-compartment minimal model for estimation the relative contribution of S_G to the glucose restoration rate during a basal state. In the basal state, healthy individuals have insulin oscillations with a regular 14-min periodicity of amplitude of 1.8 mU/L[17]. Hence, the relative contribution of S_G to the glucose restoration rate during the basal state is given by $S_G/(S_G + S_I \times 10.8)$[18]. The calculated data demonstrate that almost the total fractional glucose turnover of type 2 diabetes during the basal state results from the ability of glucose to stimulates its own uptake, *i.e.*, 89.2% (Table 3). The essence of this outcome is the rather limited impact of insulin on the reduction in glucose effectiveness, which already appears in the prediabetic phase. Of note, the reduction in S_I is essentially greater than the reduction in S_G in type 2 diabetes and its prediabetic phase, independent of the assay methods (Table 2).

The results mentioned in Table 2 touch a fundamental problem: If the insulin-sensitivity regulation system of an individual in his prediabetic or diabetic phase is unable to respond efficiently to an increase in the glucose level, what affects in this person the *non-insulin*-sensitive regulation system, *i.e.*, the reduction in the ability of glucose, per se, to stimulate its own up-take by a mass action effect and to suppress its own release? The question is: Is there a single, all-encompassing biochemical system between both the reduction in glucose effectiveness and insulin sensitivity? Up until now, the answer to this fundamental question cannot be found in any textbook, but is given in this review.

CELL MEMBRANES

Shulman *et al*[19] resolved in part the questions raised in the previous section by studying muscle glycogen synthesis in subjects with type 2 diabetes and matched controls by means of *in vivo* carbon-13 nuclear magnetic resonance spectroscopy[19-21]. They demonstrated that the muscle glycogen synthesis rate in subjects with type 2 diabetes was about 50% of the rate observed in healthy controls. The same group also investigated, under hyperglycaemic-hyperinsulinaemic conditions, the pathway: Transmembrane glucose transport into the muscle cell, conversion of intracellular glucose into glucose-6-phosphate, and *via* two more intermediates to glycogen synthase, which adds glucose to the glycogen polymer. They concluded that the results are consistent with the hypothesis that transmembrane glucose transport is the rate-controlling step in insulin-stimulated muscle glycogen synthesis in patients with type 2 diabetes and the delivery of insulin is not responsible for the insulin resistance. Based on these results, two options arise for explaining the significantly reduced glycogen synthesis rate in subjects with type 2 diabetes relative to healthy controls: First, type 2 diabetes is characterized by a reduction in the amount of glucose transporter 4 (GLUT4) per cell surface area, or second, a change in the three-dimensional (3D) structure of GLUT4, which affects the amount of transmembrane glucose-transport. Northern blot and slot blot study results of biopsies of skeletal muscles obtained from individuals with type 2 diabetes and age-matched and body-weight-matched healthy controls indicated that there was no significant alteration in the level of GLUT4 mRNA and GLUT4 protein in individuals with type 2 diabetes

Table 2 Values of glucose effectiveness and insulin sensitivity[1] for minimal models

Units	Control subjects	Type 2 diabetes	*P* value	Δ (%)	Compartment	Tracer	Ref.
S_G							
min^{-1}	0.016 ± 0.001	0.010 ± 0.001	< 0.01	37.5	One	No	[13]
	0.020 ± 0.002	0.013 ± 0.001	< 0.05	35.0	One	No	[14]
	0.023 ± 0.012	0.016 ± 0.009	< 0.001	30.4	One	No	[15][2]
h^{-1}	1.20 ± 0.16	0.81 ± 0.11	< 0.001	32.5	One	No	[16]
	0.41 ± 0.04	0.33 ± 0.02	< 0.001	19.5	Two	^{13}C	[16]
	0.52 ± 0.05	0.37 ± 0.02	< 0.001	28.8	Two	^{2}H	[16]
S_I							
$10^{-4} \cdot min^{-1} \cdot (mU/L)^{-1}$	11.8 ± 2.6	6.7 ± 0.8	< 0.01	43.2	One	No	[14]
	13.45 ± 11.12	5.31 ± 3.98	< 0.01	60.5	One	No	[15][2]
$pmol \cdot L^{-1} \cdot (h^{-1})$	0.0062 ± 0.0006	0.0019 ± 0.0006	< 0.01	69.4	One	No	[16]
	0.0082 ± 0.0012	0.0036 ± 0.0006	< 0.001	56.1	two	^{13}C	[16]
	0.0098 ± 0.0013	0.0042 ± 0.0008	< 0.001	57.1	Two	^{2}H	[16]

[1]We used the conversion factor: 1 mU/L = 6.00 pmol/L;
[2]More than 10 years before the development of type 2 diabetes. S_G: Glucose effectiveness; S_I: Insulin sensitivity.

compared to healthy controls. GLUT1 mRNA and protein concentrations were also not significantly different in individuals with type 2 diabetes compared to control subjects[22,23]. This excludes the first option. To demonstrate the second option plays a pivotal role in the onset of type 2 diabetes, we must enter the area of cell membranes.

Phospholipid bilayers form rapidly and spontaneously when phospholipids are added to water. Mammalian phospholipids contain a 1,2-diacylglycerol backbone that has a phosphate group esterified at carbon atom 3, and generally a saturated fatty acid (FA) esterified at carbon atom 1, and a saturated, monounsaturated or polyunsaturated FA esterified at carbon atom 2. The two acyl chains yield a roughly cylindrical molecule that can easily pack in parallel arrays to form extended sheets of membranes composed of a mosaic of proteins and phospholipids in a fluid phospholipid matrix[24]. The driving force of this aggregation is the weak, noncovalent bond (van der Waals force) between pairs of carbon atoms, lying next to each other in the carbon 1 and the carbon 2 acyl chains. The most structural result obtained from X-ray scattering analyses of oriented bilayers in artificial phospholipid membrane systems is the area (A) per lipid molecule. This area denotes the cross-section of the cylindrical space occupied by a phospholipid. Various studies of fully hydrated, fluid phase, model phosphatidylcholine bilayers have demonstrated that introducing one or more carbon-carbon *cis* double bonds into saturated acyl chains will increase the cross-section area A (Table 4)[25-28]. The advantage of this type of artificial bilayer model is its flexibility, the ability to bend or to be bent easily without breaking.

Based on the published data, we are able using the Lennard-Jones equation: $U = (11.5 \times 10^{-6})/r^{12} - (5.96 \times 10^{-3})/r^{6}$ to estimate roughly the interaction energy between a pair of carbon atoms, which lie next to each other in the phospholipid acyl chains, esterified at the 1- and 2-positions of glycerol (Table 4)[29]. For instance, a comparison of the cross-section area (A) of an artificial bilayer consisting of dimyristoylphosphatidylcholine [(C14:0;C14:0)PC; area (A) = 60.6 $(Å)^2$] with the cross-section area (A) of an artificial bilayer consisting of palmitoyl-docosahexaenoic phosphatidylcholine [(C16:0; C22:6)PC; area (A) = 74.8 $(Å)^2$] reveals that the cross-section area (A) of the latter increases by 23.4%, which results in an increase in the interchain carbon-carbon distance of 11.2%, and thereby reduces with 37.6% the interaction energy per pair of acyl chain carbon atoms. In other words, a reduction in carbon-carbon double bonds in phospholipid acyl chains results in a reduction in membrane flexibility[30]. More generally, the flexibility of polyunsaturated phospholipids along the membrane normal (*z* direction) might soften various mechanical stresses in the membrane[31]. In other words, the number of double bonds (= unsaturation) in the acyl chains of phospholipids influences the physical properties of cellular membranes.

The degree of membrane flexibility is expressed in unsaturation index (UI) (number of carbon-carbon double bonds per 100 acyl chains) of membrane phospholipids, as observed in erythrocytes. For information about the analytical details of the lipid extraction from erythrocytes, the fatty acid analysis by gas chromatography and the calculation of UI see section Supplementary material. The UI is a variable unit; a value

Table 3 Two-compartment minimal model analysis of the relative contribution of glucose effectiveness to the glucose restoration rate of type 2 diabetes during basal state

	Units	^{13}C	^{2}H
S_G	h^{-1}	0.33 ± 0.02	0.37 ± 0.02
S_I	$h^{-1} \cdot (pmol/L)^{-1}$	0.0036 ± 0.0006	0.0042 ± 0.0008
$S_I \times 1.8 \times 6$	h^{-1}	0.0389 ± 0.0056	0.0454 ± 0.0086
$S_G/(S_G + S_I \times 10.8)$	%	89.4	89.1

S_G: Glucose effectiveness; S_I: Insulin sensitivity.

on the right of the reference interval (the range of values that is deemed normal for a physiologic measurement in healthy persons) means an acyl composition of phospholipids with an increased number of carbon-carbon double bonds, whereas a value on the left of the reference interval means an acyl composition of phospholipids with a decreased number of carbon-carbon double bonds. Borkman *et al*[5] demonstrated in the phospholipid fraction of a *vastus lateralis* muscle biopsy of healthy man that insulin sensitivity was positively correlated with the percentage of arachidonic acid in muscle (r = 0.76, P < 0.01), the total percentage of C20-C22 polyunsaturated fatty acids (r = 0.76, P < 0.01), and the unsaturation index (r = 0.62, P < 0.05).

The consequence of the aforementioned data is that a reduction in UI is associated with a reduction in insulin sensitivity, which suggests that changes in the acyl composition of tissue phospholipid membranes modulates the action of insulin. Borkman *et al*[5] suggested that if the action of insulin depends on the acyl composition of muscle membranes, it may be due to interactions within membranes specifically involved in the action of insulin, although a more general effect of membranes cannot be excluded. However, not only a reduction in insulin-mediated glucose disposal is a marker of type 2 diabetes, but also a reduction in non-insulin-mediated glucose disposal (Table 2).

It is interesting to note that the experimental results of Min *et al*[32,33] demonstrated that a key feature of the prediabetic phase, which appears in individuals with impaired glucose tolerance and women with gestational diabetes mellitus, is an essential reduction, compared to healthy controls, in the percentage of phospholipid poly-unsaturated acyl chains, including UI (Table 5, Supplementary Tables 1 and 2)[32,33]. Because phospholipid bilayers with an acyl composition of more carbon-carbon double bonds are more flexible than those with less carbon-carbon double bonds (Table 4), a reduction in membrane flexibility is a key factor regarding the increase in the plasma glucose concentration in type 2 diabetes and its prediabetic phase[34,35].

Variations in the acyl composition of phospholipid membranes can strongly influence the function of proteins embedded therein[36]. The biochemical and physical background of this mechanism is a reduction in UI, which is equivalent to a reduction in carbon-carbon double bonds of membrane phospholipids and results in a reduction of area A of the lipid molecules (Table 4). A reduction in area A translates into increased attractive forces between the mutual phospholipid acyl chains, which forms a redistribution of the lateral pressure in cell membranes. As a consequence, the redistribution induces a cross-sectional contraction of all Class I GLUT proteins, which in turn, causes a reduction in the amount of transmembrane transported glucose (Figure 1). This important hypothesis is in line with observations presented in biophysical and structural studies, which indicate that interactions of membrane proteins with lipid molecules are critical to their folding and stability[37-39]. This is what evolution is all about: Communicate with the environment and react to changes in the most efficient way[40].

The high glucose environment in women with gestational diabetes mellitus disappears after birth, in conjunction with the raised levels of free fatty acids (FFAs). So, the reduction in membrane flexibility is reversible and the restoration of UI will repair the amount of transmembrane glucose-transport. This is a nice example of how science works in unexpected ways. This generally unknown system of glucose regulation through changes in the acyl composition of phospholipids works fully independent of the insulin level and represents a beautiful unification of insulin sensitivity with glucose effectiveness. I will argue later on that this glucose-regulation system is effective in type 2 diabetes.

Several studies published UI values and supported its utility as a crucial parameter for membrane flexibility, even if still not properly considered by the scientific medical community[41]. It is interesting to note that the elegant study of Borkman *et al*[5] sums

Table 4 Experimental data of fully hydrated fluid phase phosphatidylcholine lipid bilayers

	DLPC	DMPC	DPPC	DOPC	PDPC
Reference	25	25	26,27	26	28
Fatty acid structure	$[C12:0]_2$	$[C14:0]_2$	$[C16:0]_2$	$[C18:1]_2$	C16:0;C22:6
Temperature (°C)	30	30	50	30	30
Area A per lipid molecule $(Å)^2$	63.2	60.6	64.0	72.5	74.8
Carbon interchain distance (Å)	4.49	4.39	4.51	4.80	4.88
Interaction energy U (kJ/mol)		-0.607			-0.379
UI	0	0	0	100	300

DLPC: Dilauroylphosphatidylcholine; DMPC: Dimyristoylphosphatidylcholine; DPPC: Dipalmitoylphosphatidylcholine; DOPC: Dioleoylphosphatidylcholine; PDPC: Palmitoyl-docosahexaenoic-phosphatidylcholine; UI: Unsaturation index.

up the idea that the acyl composition of skeletal-muscle phospholipids may influence the action of insulin; unfortunately, this idea turns out not to be true. The author's idea is that in type 2 diabetes a redistribution of the lateral pressure in cell membranes results in a reduction in the cross-sectional area of all class I GLUTs, and thereby reduces the transmembrane glucose-transport.

MEMBRANE FLEXIBILITY

Membrane flexibility plays an important role in the scientific understanding of the molecular nature of life. Its role is evident in type 2 diabetes for at least three reasons.

It first affects the insertion of GLUTs into a plasma membrane. GLUT1 is a monomeric protein with 12 transmembrane helical segments[42]. The transporter protein traverses the plasma membrane 12 times in a zigzag fashion before initiating the folding, which is essential for creating its final 3D structure. A central channel across the protein communicates the extracellular and intracellular environments. Several amino acid residues of GLUT1, crucial for transport of β-D-glucose, bound a channel segment of approximately 15 Å long and 7 Å wide. In the proposed structure of GLUT3 the 12 transmembrane helices form a right-hand barrel with a central pore, which is shaped like a funnel with dimensions of approximately 5-6 Å by 8 Å at its narrowest point[43]. To get an idea of the dimensions: The dimensions of an orthorhombic bisphenoidal α-glucose crystal are: a = 10.36 Å, b = 14.84 Å, and c = 4.97 Å[44]. One GLUT1 molecule with a mean cross-section area of about 1100 $Å^2$ covers an area of about 17 phospholipid molecules of a phosphatidylcholine bilayer with saturated acyl chains[27]. So the folding mechanism of a GLUT1 molecule requires flexibility of the cell membrane for achieving a correct 3D structure. In contrast, GLUT4 is inserted into membranes of intracellular vesicles, which demands flexibility of the vesicular membrane. After the insulin-stimulated translocation of the intracellular GLUT4-containing vesicles to the plasma membrane, the GLUT4 containing vesicles take part in a fusion process with the plasma membrane. In the final stage of this process, fusion proteins induce bending of the plasma membrane bilayer to drive fusion pore formation. This includes that in type 2 diabetes the transmembrane glucose transport through non-insulin sensitive glucose transporters is being hampered solely by a reduction in the plasma cell membrane flexibility, whereas the transmembrane glucose transport through the insulin sensitive glucose transporter GLUT4 is being hampered by a reduction in the flexibility of two membranes, *i.e.* the vesicular membrane and the plasma membrane[45,46]. For this reason, the reduction in S_I is essentially greater than the reduction in S_G at type 2 diabetes (Table 2).

Next, membrane flexibility is associated with atherosclerosis, a condition where arteries become narrowed and showed an increase in vascular stiffness[47]. The erythrocyte membrane is compositionally very similar to the vascular endothelium, a thin layer of cells that keeps arteries smooth and allows blood to flow easily[48]. In support of this, the UI of red cell membrane phospholipids of healthy controls was found to be 155.4[49], and the reported UI of cultured endothelial cells from human umbilical cord veins was found to be 148.2 ± 6.3[50]. So we may suggest that the UI is a highly sensitive sensor for cellular membrane functionality, *i.e.* if the erythrocyte membrane is affected in type 2 diabetes, then the endothelium may also be affected[51,52]. An amazing example of endothelial dysfunction is presented in a Watch

Table 5 Erythrocyte acyl composition of phospholipids and unsaturation index of control individuals, individuals with impaired glucose tolerance, and individuals with gestational diabetes mellitus[1]

	Biochemical characteristics	IGT			GDM		
		Control persons (*n* = 42)	Persons with IGT (*n* = 28)	Δ (%)	Control persons (*n* = 61)	Persons with GDM (*n* = 53)	Δ (%)
PC							
	Total SFAs (%)	46.4	49.2	+ 6.0	42.5	48.3	+24.6
	Total MUFAs (%)	17.8	19.1	+7.3	13.1	15.8	+20.6
	Total PUFAs (%)	26.8	22.3	-12.5	37.2	31.1	-16.4
	UI	92	79.5	-13.6	148.1	114.8	-22.5
PE							
	Total SFAs (%)	38.4	40.5	+5.5	24.6	27.2	+10.6
	Total MUFAs (%)	27.5	30.1	+9.5	18.6	20.1	+8.1
	Total PUFAs (%)	34.6	29.4	-15.0	37.8	33.7	-10.8
	UI	167.4	147.1	-12.1	177.6	159.4	-10.2

[1]Ex-post calculations performed by the author are based on the original data listed by Min *et al*[32,33]. The calculations of the biochemical characteristics are shown in Supplementary Tables 1 and 2. UI: Unsaturation index; IGT: Impaired glucose tolerance; GDM: Gestational diabetes mellitus; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; SFA: Saturated fatty acid; MUFA: Mono-unsaturated fatty acid; PUFA: Poly-unsaturated fatty acid.

WebMD Video, entitled "How atherosclerosis plaque forms"[53]. This video underlines that although the exact causes of atherosclerosis are not yet clear, many scientists think that plaque formation begins with damage of the endothelium. The author's idea is that individuals with a low UI (increased membrane stiffness) are more prone to develop atherosclerotic cardiovascular disease, compared to healthy controls.

Finally, membrane stiffness induces tissue hypoxia[54]. The minimum lumen of capillary vessels is about 4-9 μm, and the size of erythrocytes is approximately 8 μm. In the case of type 2 diabetes, the reduction in flexibility of both the erythrocyte membrane and the endothelium has a profound impact on the microcirculation[55]. The resulting decrease in blood flow leads to a reduction in oxygen supply of the surrounding tissues. Because the electrons of the respiratory chain are finally donated to molecular oxygen to form H_2O, a status of hypoxia results in an accumulation of electrons in the respiratory complexes, which finally results in the production of the negatively charged superoxide radical $O_2^{\bullet -}$ (the dot means a single unpaired electron), and thereby reduces, among other things, ATP synthesis[56]. Life can only exist by the grace of ATP synthesis. The aforementioned reduced ATP synthesis could be the principle reason that type 2 diabetes is linked to lower life expectancy[57]. The paragraph entitled "Energy transport" later on goes into more detail about the influence of type 2 diabetes on the production of ATP.

EFFECTS OF TEMPERATURE ACCLIMATION ON PHOSPHOLIPID MEMBRANE COMPOSITION

The adaptation of an integrated bilayer system to an environmental factor such as temperature is referred to as homeoviscous adaptation. Studies with regard to this theme consistently reported that cold acclimation generates an increase in polyunsaturation of cell membranes of aquatic organisms[58,59]. To keep a flexible and effective membrane at low temperatures, aquatic organisms actualize an increased concentration of unsaturated acyl chains in phospholipids relative to those at warmer temperatures (Table 6, Supplementary Table 3)[60]. It is important to note that the homeoviscous adaptation is a reversible process and exists as early as the beginning of the Ordovicium, about 500 to 400 million years ago.

Hanssen *et al*[61] reported that 10 d of cold acclimation (14-15 ˚C) markedly increased peripheral insulin sensitivity by about 43% in eight type 2 diabetes subjects. Cold acclimation resulted in an enrichment of GLUT4 at sarcolemma, which facilitated the uptake of glucose. The GLUT4 translocation could not be explained by AMPK activation or improved insulin signaling. Presumably, the authors were unaware that cold acclimation generates an increase in polyunsaturation of cell membranes, which increases membrane flexibility and reduces the cross-sectional contraction of the area

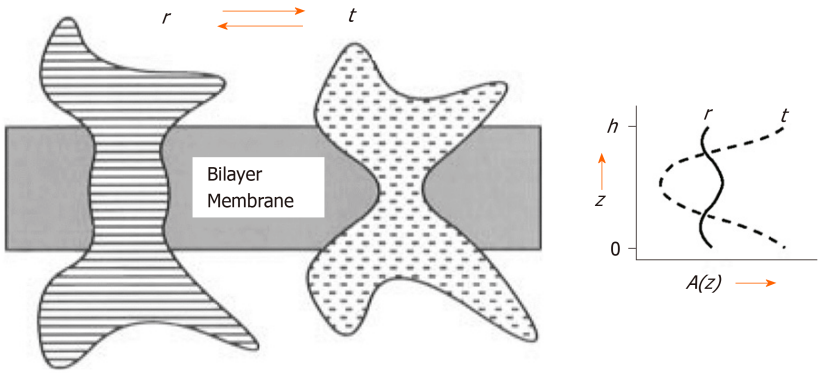

Figure 1 Slice through a bilayer membrane containing an intrinsic protein viewed in two different conformational states, *r* and *t*. At right, the cross-sectional area profile *A*(*z*) of each of the two states is plotted as a function of depth *z* within the membrane of thickness *h*. This figure is a reprint from Cantor's work[36]. Reproduced with permission from Lateral pressures in cell membranes: a mechanism for modulation of protein function. Copyright 1997 American Chemical Society.

A of all Class I GLUT proteins.

A telling example of homeoviscous adaptation is a form of evolutionary heat acclimation, which lies hidden in the relationship between the mammal body mass (M, g) and the basal metabolic rate (BMR; mL of O_2 per hour) expressed in the allometric equation of the form: BMR = 4.12 × $M^{0.69}$[62]. This relationship, with an allometric coefficient of 0.69, means that the BMR grows at a slower rate than the body mass, referred to as the slow-down principle[63]. To understand the consequences of the slow-down principle, imagine a multi-celled development of a single-celled, cube-shaped eukaryote, which grows through cell division with a same speed in all three directions of a Cartesian coordinate system. This type of growth creates a generation sequence of cube-shaped eukaryotic cells with a one cell extension in each three dimensions, per new generation (Table 7). Moreover, each unit cell burns continuously food in oxygen and the molecular remains of the food are finally converted into ATP and heat. To maintain an adequate cell temperature, each of the one-unit cells exchanges one metabolic heat unit per time unit with the environment. The first eukaryotic cell thus exchanges with the environment one heat unit per time unit through 6 identical surface planes. The next generation with 8 (2^3) unit cells and a total of 24 (6 × 2^2) unit surface planes exchanges 8 (2^3) heat units per time unit. The subsequent generation with 27 (3^3) unit cells and a total of 54 (6 × 3^2) unit surface planes exchanges 27 (3^3) heat units per time unit, and so on (Table 7).

The important outcome of this model with the growing cube-shaped eukaryotic cells is that the number of heat units to be exchanged per time unit increases by its cube, and the number of unit surface planes increases by its square (Table 7)[63]. This means that the relative rate of heat production must fall as the number of cells gets larger. How did the evolutionary trajectory of live resolve this problem? Well, over evolutionary time, the slower rate of heat production was achieved by a reduction in UI of membrane phospholipids. Hulbert *et al*[64] reported that the UI of phospholipids from mammalian species significantly decreased as species body size increased whilst the percentage of total unsaturated acyl chains was relatively constant in mammalian species of very different body mass. So the membrane bilayers of small mammals were generally high in docosahexaenoyl (C22:6 n-3) chains and low in oleyl (C18:1 n-9) chains, and the opposite was observed in large mammals[64]. A telling example of body core temperature regulation during the evolution period from mouse to *Homo sapiens* is the reduction in skeletal muscle percentage of docosahexaenoyl (C22:6 n-3) chains from approximately 30% to 2% in parallel with a body mass increase from approximately 10 g to 85.000 g[64]. Apart from the brain, the phospholipids of heart, skeletal muscle, kidney, and liver tissues showed a significant negative relationship between the body mass of the species and the docosahexaenoate (C22:6 n-3) content of tissue phospholipids. The brain phospholipids from mammals have a high and relatively constant docosahexaenoate content, irrespectively of the body size of the species[65]. Also the mass-specific metabolic rate of birds depends on the relative balance between mono-unsaturated and poly-unsaturated acyl chains. These data suggest that the biochemical translation of the slow-down principle is a replacement of polyunsaturated acyl chains by monounsaturated acyl chains in membrane phospholipids, which means that the number of unsaturated acyl chains remains the same, whereas their number of carbon-carbon double bonds decreases, with a

Table 6 Acyl composition (% of total acyl chains) of membrane phospholipids and unsaturation index in fathead minnow (Pimephales promelas) muscle[1]

Biochemical characteristics	15 ˚C	25 ˚C	30 ˚C
Total SFAs (%)	17.8	19.9	38.4
Total MUFAs (%)	16.1	16.1	26.6
Total PUFAs (%)	65.7	61.5	34.9
UI	349.9	325.9	189.9

[1]Ex-post calculations of the biochemical characteristics are performed by the author and based on the original data listed by Fadhlaoui *et al*[60]. The calculations of the biochemical characteristics are shown in Supplementary Table 3. UI: Unsaturation index; SFA: Saturated fatty acid; MUFA: Mono-unsaturated fatty acid; PUFA: Poly-cis-unsaturated fatty acid.

consequent reduction of the area A of lipid molecules with all the consequences that entails (see previous section). Instead of insulin action, heat production may cause a reduction in the amount of transmembrane glucose-transport.

Today, a human parallel of this evolutionary principle still occurs during a pregnancy, *i.e.* an increase in maternal mass may generate a reduction in UI of the acyl composition of maternal phospholipids, which in turn, lowers the maternal membrane flexibility, and thereby reduces the maternal transmembrane glucose-transport. In this way, the maternal plasma glucose concentration and insulin level increase during pregnancy without any impaired sensitivity of tissue to the action of insulin[66].

FREE FATTY ACIDS

Type 2 diabetes and its prediabetic phase are characterized, among other things, by an increase in the plasma FFA concentration[67,68]. The cause of this phenomenon is a reduction in the transmembrane glucose-transport of all Class 1 GLUTs, which leads to a reduction in the glucose-mediated ATP production. An absolute requirement for ATP production necessitates cells to make a switch from glucose-mediated ATP production to FFA-mediated ATP production – remember that a single cell consumes around 10 million molecules of ATP every second, which means that in the human body the total turnover of ATP is around 60-100 kg/d[56]. So a reduction in glucose-mediated ATP production promotes an increase in the level of essentially saturated FFAs for extra FFA-mediated ATP production, which will set up a vicious cycle of raising the levels of essentially saturated plasma FFAs and lowering the level of transmembrane glucose transport. After all, the released FFA-pool of human white cells showed approximately 110- and 9-fold decreased percentages of docosahexaenoic acid (C22:6) and arachidonic acid (C20:4), respectively, compared with the human serum pool. So, the UI of released FFAs from human white fat cells is substantially lower compared with the UI of serum FFAs in healthy controls (85.5 and 191.9, respectively; Supplementary Table 4)[69-71]. Thus, an increased release of FFAs from adipose tissue into the circulation elevates the plasma concentration of saturated fatty acids. This sequence of events forced a shift from unsaturated to saturated acyl chains in phospholipids of both the erythrocyte membrane and the vascular endothelium[68]. I am telling this matter, because I believe the existence of this vicious cycle decreases progressively the UI of plasma FFAs, which may be a root cause of the progressive character of type 2 diabetes.

ENERGY TRANSPORT

To understand the relationship between heat production and the onset of type 2 diabetes and gestational diabetes, it is time to address briefly the concept of eukaryotic cellular energy transport. Hydrogen is the energy carrier par excellence for the energy saved in our food. After absorption of food, hydrogen is being stripped from the molecular remains of the nutrients, and passed *via* the citric cycle into the mitochondrial electron-transport chain. Mitochondria are surrounded by a simple outer membrane and a more complex inner membrane. The space between these two membranes is referred to as the intermembrane space and the space surrounded by the inner membrane as the matrix. The four separate protein complexes of the electron-transport chain are located in the inner membrane. The first two complexes

Table 7 Thought experiment of multi-celled development of a single-celled, cube-shaped eukaryotic cell, which grows through cell division in a Cartesian coordinate system with the same speed in all three directions, and exchanges with its environment per unit-cell one heat unit per time unit[63]

Growth of cubic species	Number of unit cubes	Total cube	
		Required number of heat units to exchange	Number of unit-cube surface planes
Original cube	1	1	6
First generation	8 (2^3)	8 (2^3)	24 (6×2^2)
Second generation	27 (3^3)	27 (3^3)	54 (6×3^2)
Third generation	64 (4^3)	64 (4^3)	96 (6×4^2)
Fourth generation	125 (5^3)	125 (5^3)	150 (6×5^2)

separates the electrons from the hydrogen energy-carriers, after which the electrons are finally donated to molecular oxygen, the ultimate electron acceptor in complex IV of the electron-transport chain, to form H_2O. In concert with these processes, respiration pushes the remaining protons (H^+-ions) across the mitochondrial inner membrane into the intermembrane space, against a concentration gradient. These protons could re-enter the matrix through the inner mitochondrial membrane in two different ways, first *via* the channel of the ATP synthase protein complex for driving ATP synthesis, and second *via* the uncoupling protein1 (UCP1) without using energy for any purpose. In the last way, the proton potential energy is released as heat[72]. It is therefore concluded that UCP1 may play a crucial role in thermogenesis.

HEAT PRODUCTION AND THE ONSET OF TYPE 2 DIABETES

Experimental data of intracellular temperature mapping, based on a novel fluorescent polymeric thermometer and fluorescence lifetime imaging microscopy, demonstrated clearly the existence of mitochondrial-mediated heat production[73]. This heat production was observed as a proximal local temperature increase. It could be concluded that the local temperature near the mitochondria was higher than the temperature of the rest of the space in the cytosol (aside from the centrosome). Furthermore, this local heat release from mitochondria is accelerated when ATP synthesis is stalled by an uncoupling reagent[74]. Despite incomplete understanding of the uncoupling functions for maintaining energy homeostasis, all these data suggest that, in healthy subjects, a balance exists between the amount of protons, which re-enter the matrix through ATP synthase on the one hand, and the amount of protons, which re-enter the matrix through UCP1 on the other.

The reviewed data strongly support an alternative model with proposed steps in the development of type 2 diabetes, a disease arising as a result of a hypothetical hereditary anomaly (Figure 2)[63]. The author's hypothesis proposes that the final result of this anomaly, which already appears in the prediabetic phase, is a status of an increased flux, compared to healthy controls, of intermembrane-space protons, which re-enter the matrix *via* UCP1, and thereby causes hyperthermia in and around the mitochondria[63]. To keep the mitochondrial temperature within the narrow range compatible with live, the slow-down principle enters into force, which results in an appreciable reduction in UI. This process leads to a marked reduction in membrane flexibility, and thereby reduces the transmembrane glucose-transport, which generates a reduction in glucose-mediated heat production. However, the concomitant disadvantage of this sequence of events is also a reduction in glucose-mediated ATP production. To compensate for the extra loss of glucose-mediated ATP, lipolysis increases to raise the levels of circulating, essentially saturated FFAs, which are needed to generate extra ATP energy for sustaining life. The progressive reduction in UI will set up a vicious cycle of raising the levels of essentially saturated plasma FFAs and lowering the level of transmembrane glucose transport. These phenomena represent a blueprint of the presence of type 2 diabetes and its progressive character in human individuals.

Remember that Kelley *et al*[75] reported the presence of impaired functional capacity and morphological alterations of mitochondria, which were obtained from the vastus lateralis muscle of volunteers with type 2 diabetes. A status of long-term heat acclimation may be the cause of the reported reduced activity of NADH oxidation by the respiratory chain, the smaller mean size of mitochondria with a less clearly defined internal membrane structure, and smaller cristae.

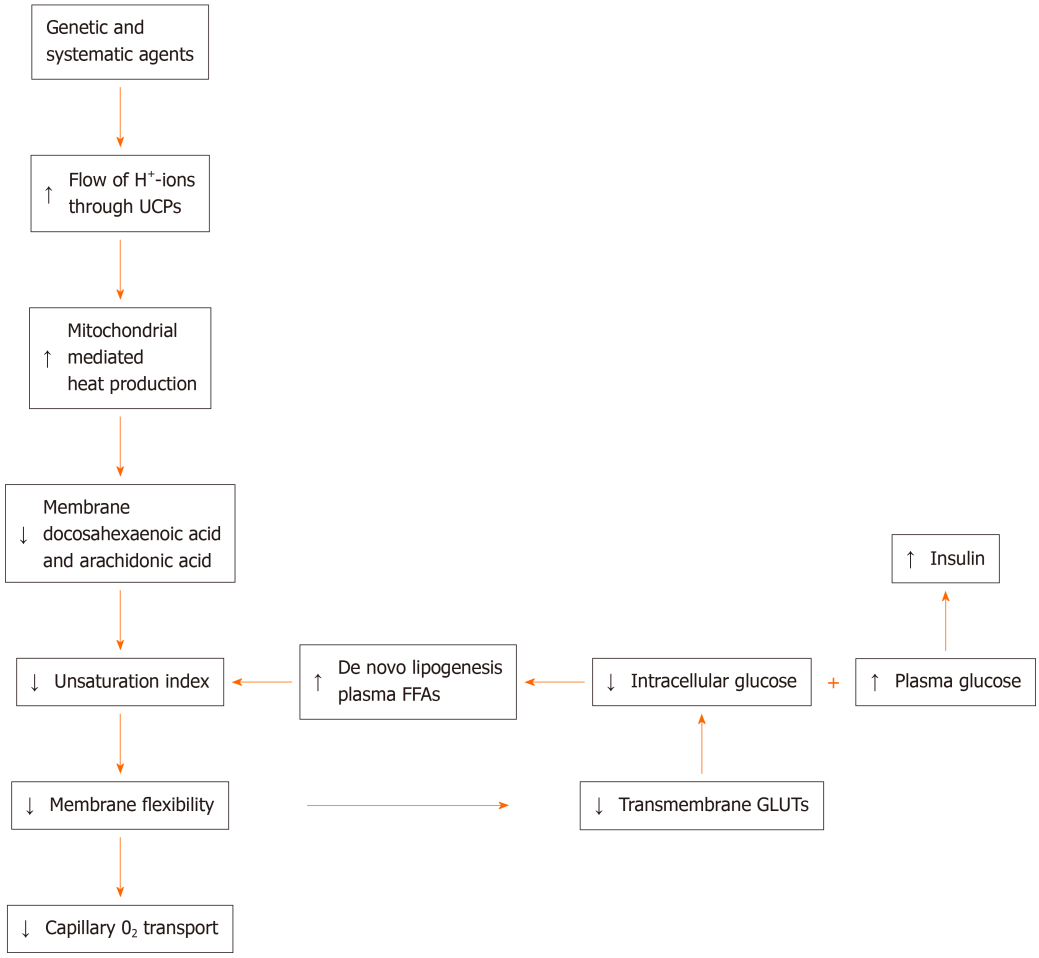

Figure 2 Although the results of genome-wide screen for type 2 diabetes susceptibility genes are still under debate, a refined working hypothesis proposes that the primary effect of the involved genes generates an increased flux of mitochondrial intermembrane-space protons through UCP1 into the matrix, which causes an increase of extra heat. This process initiates the slow-down principle. UCP: Uncoupling protein; FFA: Free fatty acid; GLUT: Glucose transporter.

We can now explain why the classical research results of laboratory animals mimicking the human type 2 diabetes has not given any indication regarding the onset of human type 2 diabetes[76,77]. All in all, the characteristics of the animal model should mirror the pathophysiology and natural history of type 2. By contrast, no experimental results are given so far based on animal models with type 2 diabetes, caused by a status of long-term heat acclimation.

METFORMIN

The proposed steps in the development of type 2 diabetes model improves our knowledge of the metformin-medication effects[63]. The most widely accepted model of the metformin antihyperglycemic action is its suppression of gluconeogenesis with the amino acid alanine as glucogenic substrate, which principally occurs as a consequence of mitochondrial inhibition[78]. In this context it is interesting to note that the type 2 diabetes specific reduction in the transmembrane glucose-transport promotes a significantly increased hepatic efficiency, compared to healthy controls, in converting alanine to glucose[79,80]. The suppression of hepatic gluconeogenesis creates a disturbed participation of glucose in the supply of ATP energy to the body and includes, among other things, stimulation of lipolysis. This process increases the amount of circulating, essentially saturated FFAs, referred to as metformin-mediated FFA increase. Besides the increase of essentially saturated FFAs due to the FFA-mediated ATP production (see section: Free fatty acids), the metformin-mediated FFA-increase could also have a deleterious effect on the membrane flexibility, which accelerates the onset of vascular and neurological complications over the long term[81]. The results of two studies are in line with this idea. First, the Diabetes Prevention Program (DPP) study results indicated that metformin therapy was not as beneficial

as life style modification for delaying the development of type 2 diabetes in individuals at high risk of type 2 diabetes, *i.e.* the DPP study results demonstrated that lifestyle intervention was twice as good as metformin therapy for delaying the development of type 2 diabetes, and at least as effective in older participants as it was in younger participants[82]. Second, the saturated acyl chain percentages in erythrocyte membrane phospholipids of control individuals, people with type 2 diabetes without retinopathy, and people with type 2 diabetes with retinopathy increased within a lifetime from 42.0% *via* 44.2% to 46.9%, respectively, (Table 8, Supplementary Tables 5 and 6)[83]. However, the percentage of the saturated acyl content of membrane phospholipids was almost constant in mammalian species, independent of their body mass, during the last 600 million years of evolution, which means that a constant percentage of membrane saturated acyl chains is important for biological and biochemical processes[62]. Basically, metformin reduces the plasma glucose concentration, but tends towards an increase in the FFA concentration. This side-effect of metformin is not limited to metformin, but also applies to another class of drugs, which are used for lowering the plasma glucose concentration *via* inhibiting the glucose reabsorption by inhibiting the sodium-glucose co-transporter[84].

It is worth to note that the patients included in the "diabetic retinopathy" study were treated according to the recommendations of the French High Authority of Health, who published its last recommendations with metformin as the optimal first-line drug, for the optimal management of diabetes, on January 2013 (personal communication, Niyazi Acar)[85].

EXERCISE

Although the role of irisin in the conversion of white adipose tissue into brown adipose tissue is still under debate[86], acute exercise training showed direct effects on "browning" of white fat[87]. Hamilton *et al*[88] demonstrated in excised murine adipose tissue samples lower levels of unsaturated triglycerides in brown adipose tissue compared with white adipose tissue, an observation consistent with previous results[89]. If the gene expression in the corresponding mouse model does not significantly differ from the human conditions[90], burning of human brown adipose tissue, due to physical activities, induces in phospholipids a shift from saturation into unsaturation, which promotes membrane flexibility. Also, muscular exercise increases the blood flow[91], which promotes oxygenation of cells, stimulates the electron flow down of the respiratory chain, and improves the proton (H^+) flux through the ATP synthase driving ATP synthesis, which in turn, reduces the need for FFA-mediated ATP production, and thereby increases membrane flexibility. This may be the main cause, why the US DPP study results indicated that the incidence of type 2 diabetes was 58% lower in the lifestyle-intervention group than in the placebo group[82]. Cellular flexibility is a critical factor for modulating blood flow in microcapillaries (see section: Membrane flexibility). So, an increase in both erythrocyte-membrane flexibility and microvascular endothelium flexibility is of unprecedented clinical relevance: It generates a beneficial reduction in microvascular and macrovascular complications,

Structured lifestyle intervention trials (including physical activity, dietary energy restriction and weight loss) demonstrated reductions of 28%-59% in the risk of developing type 2 diabetes in individuals with impaired glucose tolerance[82,92,93]. However, it is important to note that all the exercise sessions in these randomized controlled trials were supervised, a situation that is not often met in daily practice.

CONCLUSION

This review highlights a deeper scientific understanding of the molecular nature about important factors underlying the pathophysiology of type 2 diabetes and its prediabetic phase. Work described in this review is designed to communicate some newly discovered fundamental biomolecular processes governing this disease to a broad audience including everyone who is involved in the complex field of this disease, which is one of the today's greatest unsolved medical mysteries.

The discovery of the existence of yet another blood glucose regulation mechanism is a milestone in the literature about type 2 diabetes. This overlooked transmembrane glucose transport mechanism, still not mentioned in the medical literature, is based on a redistribution of the lateral pressure in cell membranes, due to a reduction in membrane flexibility, which influences the 3D structure of all Class I GLUT proteins. Variations in the number of carbon-carbon double bounds of phospholipid acyl

Table 8 Erythrocyte acyl chain composition in phospholipids and unsaturation index of control individuals, people with type 2 diabetes without retinopathy, and people with type 2 diabetes with retinopathy[1]

Biochemical characteristics	Control individuals (*n* = 18)	Individuals with type 2 diabetes without retinopathy (*n* = 14)	Individuals with type 2 diabetes with retinopathy(*n* = 46)
Total SFAs (%)	42.0	44.2	46.9
Total MUFAs (%)	18.8	21.7	21.3
Total PUFAs (%)	38.0	31.9	29.5
UI	155.4	134.3	123.3

[1]Ex-post calculations performed by the author (see appendix) are based on the original data listed by Koehrer *et al*[83]. The calculations of the biochemical characteristics are shown in Supplementary Tables 5 and 6. SFA: Saturated fatty acid; MUFA: Mono-unsaturated fatty acid; PUFA: Poly-unsaturated fatty acid; UI: Unsaturation index.

chains of fluid lipid bilayers modulates the GLUT protein pore diameter of the transmembrane glucose transport channel. In short, impaired glucose tolerance, gestational diabetes mellitus and type 2 diabetes are characterized by an elevated concentration of essentially saturated FFAs, which creates less flexible membranes, narrows the glucose channels of all Class I GLUT proteins, and thereby lowers the amount of transmembrane glucose transport. Martin *et al*[15] reported that 10 years before the development of type 2 diabetes glucose effectiveness and insulin sensitivity in normoglycaemic offspring of couples, who both had type 2 diabetes, were 30% and 60%, respectively, lower compared to controls. This fact indicates that a redistribution of the lateral pressure in cell membranes, due to a reduction in membrane flexibility, influences the 3D structure of all Class I GLUT proteins early in live, which is an unprecedented discovery.

A second milestone is the observation that a reduction in UI, compared to a healthy status, is a characteristic of both the prediabetic phase and diabetic phase of type 2 diabetes. In a previous study I offered the clue that a raised leakage of H^+-ions *via* UCP1 induces in and around mitochondria hyperthermia, which will lead to both a reduction in mitochondrial activity and content[94]. The proposed hyperthermia is countered by the slow-down principle already in the prediabetic phase and initiates a status of long-term heat acclimation, which generates a reduction in both insulin sensitivity and glucose effectiveness, and an increase in the percentage of the saturated acyl content of phospholipids. Both milestones degrades "insulin resistance" into an imaginary reality and requires a U-turn in thinking on some important issues of type 2 diabetes.

The genetic cause of hyperthermia remains incurable until today; however, there is a way forward. Metformin is useful to obtain a rapid reduction in (too) high glucose concentrations. Doctors who prescribe metformin should give clear information to patients about the metformin-mediated FFA increase and therefore point out the necessity of exercise and diet, which will minimize this metformin side effect. They should control the effect of exercise prescription on patients' wellbeing by the assessment of plasma FFA concentration for monitoring purposes and the assessment of UI for controlling the level of the total percentage saturated acyl chains of membrane phospholipids, as observed in erythrocytes. To prevent or delay the progressive character of type 2 diabetes, exercise is the medication to be preferred, because it improves blood circulation, micro-oxygenation of cells, and thereby reduces membrane stiffness. However, given the high degree of difficulty of structured lifestyle intervention, exercise sessions need counseling, just like with dietary energy restriction[95].

Remember that the complete oxidation of palmitic acid or stearic acid to CO_2 and H_2O per hydrogen atom more molecules of ATP produces, compared to a complete oxidation of glucose[96], which logically demands less FFA molecules, compared to glucose molecules, for a similar amount of ATP production. However, against the background of the observation that the saturated acyl chain percentages in erythrocyte membrane phospholipids of control individuals, people with type 2 diabetes without retinopathy, and people with type 2 diabetes with retinopathy increased within a life-time, the conclusion is that circulating saturated FFAs play a main role in the pathophysiology of type 2 diabetes.

A detailed working hypothesis proposes that a key feature in the etiology of type 2 diabetes, which appears in the prediabetic phase, is an essentially raise, compared to healthy controls, in the flux of intermembrane-space protons, which re-enter the matrix *via* UCP1, and thereby causes hyperthermia in and around the mitochondria. The reviewed data highlight the need for studies to find the cause of the essential

raise in this proton flux through UCP1, because it will improve the possibilities for diagnosis of type 2 diabetes, treatment, and care.

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ORIGINAL ARTICLE

Basic Study

Maternal low protein diet induces persistent expression changes in metabolic genes in male rats

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Abstract

BACKGROUND

Perinatal exposure to a poor nutritional environment predisposes the progeny to the development of metabolic disease at the adult age, both in experimental models and humans. Numerous adaptive responses to maternal protein restriction have been reported in metabolic tissues. However, the expression of glucose/fatty acid metabolism-related genes in adipose tissue and liver needs to be described.

AIM

To evaluate the metabolic impact of perinatal malnutrition, we determined malnutrition-associated gene expression alterations in liver and adipose tissue.

METHODS

In the present study, we evaluated the alterations in gene expression of glycolytic/Krebs cycle genes (Pyruvate dehydrogenase kinase 4 and citrate synthase), adipogenic and lipolytic genes and leptin in the adipose tissue of offspring rats at 30 d and 90 d of age exposed to maternal isocaloric low protein (LP) diet throughout gestation and lactation. We also evaluated, in the livers of the same animals, the same set of genes as well as the gene expression of the

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transcription factors peroxisome proliferator-activated receptor gamma coactivator 1, forkhead box protein O1 and hepatocyte nuclear factor 4 and of gluconeogenic genes.

RESULTS
In the adipose tissue, we observed a transitory (*i.e.*, at 30 d) downregulation of pyruvate dehydrogenase kinase 4, citrate synthase and carnitine palmitoyl transferase 1b gene expression. Such transcriptional changes did not persist in adult LP rats (90 d), but we observed a tendency towards a decreased gene expression of leptin (P = 0.052). The liver featured some gene expression alterations comparable to the adipose tissue, such as pyruvate dehydrogenase kinase 4 downregulation at 30 d and displayed other tissue-specific changes, including citrate synthase and fatty acid synthase upregulation, but pyruvate kinase downregulation at 30 d in the LP group and carnitine palmitoyl transferase 1b downregulation at 90 d. These gene alterations, together with previously described changes in gene expression in skeletal muscle, may account for the metabolic adaptations in response to maternal LP diet and highlight the occurrence of persistent transcriptional defects in key metabolic genes that may contribute to the development of metabolic alterations during the adult life as a consequence of perinatal malnutrition.

CONCLUSION
We conclude that perinatal malnutrition relays long-lasting transcriptional alterations in metabolically active organs, *i.e.*, liver and adipose tissue.

Key words: Metabolic adaptation; Phenotype plasticity; Liver; Adipose tissue; Metabolism; Maternal protein undernutrition; Rats

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Core tip: Perinatal exposure to a poor nutritional environment predisposes to metabolic disease. Here, the expression of metabolism-related genes in adipose tissue and liver was investigated. We evaluated the alterations in gene expression of glycolytic/Krebs cycle genes, adipogenic and lipolytic genes in adipose tissue of offspring rats at 30 d/90 d of age, exposed to maternal low protein diet throughout gestation/lactation. We also evaluated expression of liver transcription factors and gluconeogenic genes. Persistent gene alterations were observed that may account for the metabolic adaptations in response to maternal low protein diet, highlighting the occurrence of persistent transcriptional defects as a consequence of perinatal malnutrition.

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INTRODUCTION

Perinatal malnutrition occurring during pregnancy and lactation not only has a negative impact on fetal development and neonatal growth but also relays long-lasting adverse effects resulting in increased susceptibility to cardiovascular and metabolic diseases in adulthood, as posited by the developmental origin of health and disease hypothesis[1,2]. Evidence from epidemiological cohorts, with the Dutch Famine Birth Cohort Study being the most relevant[3], and experimental animal models[4-6] support the idea that a poor nutritional environment during fetal and early postnatal life predisposes to cardiovascular and metabolic disease in adulthood. The occurrence of persistent epigenetic alterations has been proposed as one of the mechanisms linking *in utero* nutritional deprivation to increased risk of disease in adulthood. Individuals who were prenatally exposed to the Dutch famine during the 1944-1945

were shown six decades later to have lower DNA methylation of the imprinted *IGF2* gene in comparison to their siblings not exposed to the famine period[7]. Observational studies have suggested a link between poor fetal growth and the development of impaired glucose tolerance at the adult age in both sexes[8], and final evidence that maternal nutrition during gestation affects glucose metabolism in adult life was provided by the observation that an oral glucose load in adults exposed prenatally to the Dutch famine led to higher glycemic concentrations as compared to individuals being born around the same years but not exposed *in utero* to the 1944-1945 famine[9].

Work in animal models suggested that the link between early-life malnutrition and the increased risk of developing metabolic disease in adulthood may be mediated by persistent biochemical alterations in the main insulin-responsive tissues, including glycolytic and oxidative skeletal muscle fibers[10]. Exposure of mice to a maternal protein-restricted diet during gestation and lactation was shown to impact the morphological features and body distribution of white adipose tissue and to reduce the protein expression levels of most of the key insulin signaling proteins, including IRS1, the PI3K subunits p110 and p85, Akt1 (v-akt murine thymoma viral oncogene homolog 1) and its phosphorylated form on serine 473 in male offspring[11], resulting in an altered distribution and morphology of white adipose tissue[12]. In a similar way, perinatal low protein (LP) diet consumption contributed to fatty liver phenotype at the adult age, which the magnitude was related to the period of exposure to the LP diet[13].

The occurrence of alterations of glycemic control in humans, associated to the observation that perinatal malnutrition induces defects in the insulin signaling pathways in rodent models, prompted us to evaluate whether the main metabolic pathways are affected by a LP diet administered to dams during pregnancy and lactation. In a previous study, we reported that gene and protein expression of enzymes participating in glucose and fatty acid metabolism in skeletal muscle were altered at short-term (30 d) and long-term (90 d) timepoints in male rat offspring exposed to a maternal LP diet during gestation and lactation[10]. Interestingly, we observed, both in soleus and extensor digitorum longus skeletal muscle, a LP-induced, long-lasting downregulation of pyruvate dehydrogenase kinase 4 (*PDK4*), an enzyme leading to allosteric deactivation of the pyruvate dehydrogenase complex *via* phosphorylation and hence a redirection of the metabolic flux from catabolic Krebs cycle to anabolic pathways[14].

To obtain a wider description of the effects of perinatal LP diet on insulin-responsive tissues, the main goal of the present study was to evaluate the short-term and long-term effects of a LP diet during gestation and lactation on the expression of key genes involved in the metabolism of glucose and fatty acid in the liver and adipose tissue of male rat offspring. We demonstrate the occurrence of long lasting alterations of gene expression in both tissues, up to 90 d of age, that reflect the persistence of altered metabolism in the offspring consequent to *in utero* and early-life exposure to deleterious nutritional conditions.

MATERIALS AND METHODS

The experimental protocol was approved by the Ethical Committee of the Biological Sciences Centre (protocol 23076 062778/2014-38), Federal University of Pernambuco, Brazil. All efforts were made to minimize animal discomfort and the number of animals used; in addition, we followed the Guidelines for the Care and Use of Laboratory Animals.

Animals

Male albino Wistar rats (*Rattus novergicus*) were obtained from the Academic Center of Vitoria de Santo Antão animal facility, Federal University of Pernambuco, Brazil. Animals were housed at 22 ± 1 °C with a controlled light–dark cycle (dark 18:00–06:00 h). Standard laboratory chow (52% carbohydrate, 21% protein and 4% lipids-Labina, Purina Agriband, São Paulo, Brazil) and water were administered *ad libitum*. Groups were divided according to their mother's diet: Control pups from dams fed a 17% protein diet (n = 5, normal protein group, NP), and LP pups from dams fed an 8% casein diet (n = 5, low protein, LP) during gestation and lactation. Diets were prepared at the Laboratory of Experimental Nutrition-Center of Vitoria de Santo Antão, Federal University of Pernambuco, according to the American Institute of Nutrition–AIN-93 dietary guidelines[15].

During suckling, offspring was maintained as litters of eight pups of both sexes to ensure standardized nutrition until weaning. At weaning (21 d postpartum), three to four male offspring of each litter were housed in collective cages and received

standard diet and water *ad libitum*. The experimental groups consisted of one or two male rats from each mother. Female offspring were not included in the present study. All experimental analyses were performed in adipose tissue and liver collected from male rats sacrificed either at 30 d old or 90 d old by decapitation. All rats were euthanized between 14:00-17:00 after a 4-5 h fasting period. The liver and visceral adipose tissue were carefully dissected, snap-frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

RNA extraction, reverse transcription and quantitative PCR

Total RNA was extracted from liver and visceral adipose tissue with Tripure reagent [Sigma-Aldrich (Roche), St. Quentin Fallavier, France] according to the manufacturer's instructions. Briefly, 10 µL of Tripure per milligram of tissue was added, and the resulting suspension was homogenized using a Precellys Lysing kit (Bertin, Montigny-le-Bretonneux, France) according to the manufacturer's instructions. After grinding, 1/4 volume of chloroform was added. They were vortexed 3 × 15 s, incubated at room temperature for 5 min and centrifuged for 15 min at 15000 g at 4 °C.

RNA was precipitated by addition of 1/2 volume of isopropanol (Carlo Erba, Val-de-Reuil, France) and centrifugation (15 min at 15000 g at 4 °C). Supernatants were used for protein extraction and RNA-containing pellets were washed sequentially with 70% and 95% ethanol (Carlo Erba), dried and dissolved in 100 µL RNase-free water. RNA concentration and purity (260/280 nm absorbance ratio) was determined on a Nanodrop 2000 (Thermo-Fisher).

Reverse transcription was performed using an RT-Takara kit (Primescript TM, Takara) using 1 µg of RNA as template and following the manufacturer's instructions. Briefly, samples were heated for 10 min at 65 °C. Samples were mixed with 4 µL PrimeScript Buffer 5 ×, 1 µL oligodT (50 µM), 4 µL random hexamers and 1 µL of PrimeScript RT Enzyme Mix followed by a 15 min incubation at 37 °C and 15 s at 85 °C. RNA was removed by incubation with 1 µL of RNase H for 20 min at 37 °C. Reverse transcription reactions were brought to 200 µL final volume by adding RNase free water and stored at -20 °C. Real-time quantitative PCR (qPCR) amplification was performed using a Rotor-Gene Real-Time PCR System (Labgene Scientific Instruments, Archamps, France). Sequences of primers used in this study are available upon request.

Reactions were incubated at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C, 10 s), annealing (58-62 °C depending on the primer sets, 30 s) and elongation (72 °C, 30 s). mRNA expression levels of *PDK4*, citrate synthase (*CS*), carnitine palmitoylacyltransferase 1b, acetyl-CoA carboxylase, fatty acid synthase, leptin, insulin receptor, phosphofructokinase, beta hydroxyacyl-coenzyme-A dehydrogenase, peroxisome proliferator-activated receptor-alpha coactivator 1 alpha, peroxisome proliferator-activated receptor-alpha, forkhead box protein O1, hepatocyte nuclear factor 4, glucose 6-phosphatase, phosphoenolpyruvate carboxykinase and pyruvate kinase L/R were measured on total RNA extracted from adipose tissue and liver. qPCR results from each gene (including the housekeeping gene) were expressed as arbitrary units derived from a standard calibration curve derived from a reference sample. Reference samples were generated by mixing 10 µL aliquots from ten cDNA samples, five from the NP group and five from the LP group. qPCR for each sample was carried out in duplicate. Gene expression data were normalized using ribosomal protein L19 as a housekeeping gene. As a further control, qPCR amplicons were analyzed by agarose gel to validate the amplicon size.

Statistical analysis

Statistical analysis was conducted with GraphPad Prism 5 program for Windows (GraphPad Software®. Inc., La Jolla, CA, United States). Exploratory data analysis was used to identify possible inaccurate information and the presence of outliers and to test the assumption of normality in all data distributions. Kolmogorov-Smirnov and Shapiro-Wilk normality tests were applied in total sample. Statistical significance was evaluated using analysis of variance ANOVA two-way test with maternal diet (low/normal protein) and age (30 d and 90 d) as factors. Bonferroni's post hoc test was used. The values are presented as mean and standard error means, and P values < 0.05 were considered statistically significant. P values < 0.05 are denoted as "a" in figures; P values < 0.01 are denoted as "b" in figures.

RESULTS

Perinatal LP diet in rats programs a lower body weight in the offspring

We applied a model of perinatal protein restriction to rat dams throughout pregnancy and lactation followed by a switch to a NP diet after weaning as schematically represented in Figure 1.

In spite of the administration of a NP diet after weaning, the LP group displayed a lower bodyweight both at 30 d [NP: 106.3 ± 17.1 g, LP: 87.3 ± 6.0 g; $P < 0.05$, unpaired *t*-test, $n = 12$ (NP) and 6 (LP)] and 90 d of age (NP: 326.7 ± 22.6 g, LP: 306.2 g; $P < 0.05$, unpaired *t*-test, $n = 8$ for both groups).

Perinatal LP diet reprograms gene expression patterns in the adipose tissue

The gene expression of two key enzymes linking the glycolytic pathway to the Krebs cycle, *PDK4* and *CS*, was evaluated in adipose tissue at different ages (Figure 2).

In NP rats, *CS* and *PDK4* genes displayed a time-dependent downregulation, with mRNA expression at 90 d being lower than at 30 d ($P = 0.057$ for *PDK4* and < 0.01 for *CS*; Figure 2). In comparison, the maternal LP diet, by inducing a significant downregulation of both genes at 30 d, abolished the time-dependent downregulation of both genes. These results indicate that the glycolytic flux may be altered in LP offspring.

On the contrary, genes related to fatty acid metabolism, while showing a time-dependent downregulation with lower expression at 90 d, were not affected by the administration of a LP diet during pregnancy and lactation (Figure 3).

These results indicate that the glycolytic flux may be altered in the adipose tissue of LP offspring while fatty acid metabolism is not affected. As body weight of LP offspring remained lower at 30 d and 90 d as compared to NP offspring, we also evaluated the gene expression levels of leptin and observed a quasi-significant decrease of the hormone's gene expression ($P = 0.052$; Figure 4).

Perinatal LP diet reprograms gene expression patterns of multiple pathways in the liver

The expression of genes of the glycolytic pathway and Krebs cycle was evaluated in the liver at 30 d and 90 d (Figure 5). As observed in adipose tissue, *PDK4* expression was reduced in the LP group at 30 d. Conversely, *CS* was significantly upregulated at 30 d in the LP group but then returned to levels comparable to the NP group at 90 d. The phosphofructokinase gene and insulin receptor gene did not show any difference between the two groups.

In the liver, genes related to fatty acid metabolism were also significantly modulated by the perinatal exposure to a LP diet (Figure 6). Fatty acid synthase, that displayed a strong age-dependent downregulation in both groups, is strongly upregulated in the LP group at 30 d, but then returned to levels comparable to the NP group at 90 d. On the contrary, carnitine palmitoylacyltransferase 1b (fatty acid transporter) showed a significant age-dependent increase in the NP group and was strongly downregulated at 90 d in the LP group.

Transcriptional patterns in the liver are orchestrated by a group of key transcription factors that include peroxisome proliferator-activated receptor-alpha coactivator 1 alpha, forkhead box protein O1 and hepatocyte nuclear factor 4. None of these genes were significantly affected in the LP group, and the time-dependent decrease of the gene expression of peroxisome proliferator-activated receptor-alpha coactivator 1 alpha was maintained in both LP and NP groups (Figure 7).

The liver is the quantitatively major organ responsible for gluconeogenesis, providing glucose supply during starvation. We evaluated the impact of the LP diet on gluconeogenic genes by measuring mRNA levels of glucose 6-phosphatase and *PEPKC* without detecting any LP-induced defect. However, pyruvate kinase L/R was significantly downregulated in the LP group at 30 d, suggesting an accumulation of phosphoenolpyruvate and potentially a higher gluconeogenic rate at 30 d due to higher abundance of the precursor (Figure 8).

DISCUSSION

The developmental origin of health and disease model supports the idea that exposure in the critical periods of development, represented by the prenatal and early postnatal life, to a poor nutritional status, toxic substances, drugs or other kind of stress can predispose to the development of disease states, including metabolic syndrome and diabetes during adult life[2]. In particular, early-life undernutrition, especially when associated to a nutritional transition leading to obesity, is associated to a higher incidence of diabetes in adult life[16]. One of the mechanisms proposed to explain such long lasting effects is the development of epigenetic alterations that sustain alterations of gene expression patterns from the young age into adulthood[17,18]

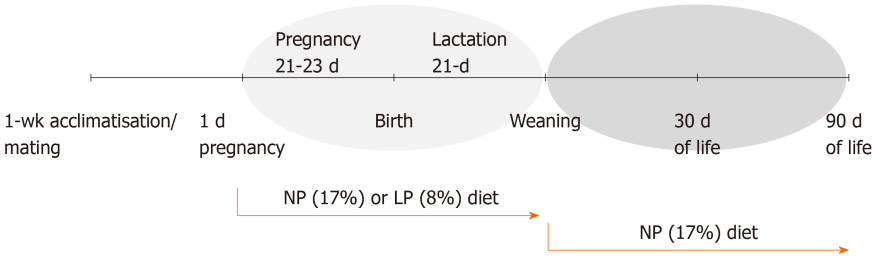

Figure 1 Schematic diagram of the experimental protocol in Wistar rats. Rats were exposed to maternal low protein diet during gestation and lactation and then switched to a normal protein diet. NP: Normal protein; LP: Low protein.

by causing persistent alterations of DNA methylation patterns, histone post-translational modifications and microRNA patterns[19,20].

In a previous study, using a rat model of perinatal protein undernutrition throughout gestation and lactation, there were changes in the gene and protein expression levels of key enzymes of glycolysis and fatty acid oxidation pathways in the skeletal muscle of the progeny, observing both postnatal acute effects (at 30 d of age) and chronic effects (at 90 d of age), the latter being representative of adaptive processes[14]. Specifically, oxidative soleus muscle responded to a LP maternal diet by downregulating hexokinase 2 and *PDK4* up to 90 d of age. For glycolytic extensor digitorum longus, the effects of a LP maternal diet were more pronounced at 30 d of age with a similar downregulation of genes coding for enzymes of the glycolytic pathway[10].

To obtain a more exhaustive description of the transcriptional disturbances induced by a perinatal LP diet in insulin-responsive tissues, we have now investigated the transcriptional changes resulting from a prenatal and postnatal exposure to LP in visceral adipose tissue and liver.

A key finding of our study is the short-term downregulation of *PDK4*, observed both in liver and adipose tissue and previously detected in soleus and extensor digitorum longus. PDK4 by phosphorylating the pyruvate dehydrogenase complex inhibits its activity and the resulting production of acetyl CoA. In the LP condition, PDK4 downregulation would therefore favor the activity of the pyruvate dehydrogenase complex and increase the glycolytic flux into the Krebs cycle[21,22].

Our observations pointing to a *PDK4* modulation in the LP diet condition underscores the central role of this enzyme in regulating metabolic flexibility, which was also observed in the heart[23,24]. Interestingly, the first enzyme of the Krebs cycle, CS appears to be upregulated in the liver, while in the adipose tissue is downregulated, thus favoring the use of newly synthesized acetyl CoA as a lipogenic substrate[21]. To keep with this hypothesis, we observed a parallel decrease, at 30 d of age, of carnitine palmitoylacyltransferase 1b, the rate-controlling enzyme of long-chain fatty acid beta-oxidation pathway. At the more advanced age of 90 d, such transcriptional changes in the adipose tissue were lost, but a decrease in the expression of the gene coding for leptin was observed, which neared statistical significance (P = 0.052, Figure 4). We hypothesize that decreased leptin gene expression observed at 90 d of age in the LP group may be a compensatory mechanism to induce higher food uptake in the LP animals as this group has a significantly lower body weight both at 30 d and 90 d of age.

The liver also shows gene expression alterations that would favor anabolic pathways. At 30 d, fatty acid synthase is upregulated in the LP group suggesting increased hepatic lipogenesis. At the same time, gluconeogenesis may also be increased in the LP group. While phosphoenolpyruvate carboxykinase and glucose 6-phosphatase were not upregulated, we observed downregulation of the pyruvate kinase L/R at 30 d, which may favor the accumulation of phosphoenolpyruvate and thus funneling of this glycolytic intermediate into gluconeogenesis.

Taken together, the gene expression changes that we have observed in the liver and adipose tissue in male rats submitted to perinatal LP undernutrition suggest the occurrence of improved lipogenesis (in adipose tissue) and gluconeogenesis (in liver) that may provide a compensatory effect to counteract the early-life exposure to the perinatal LP diet.

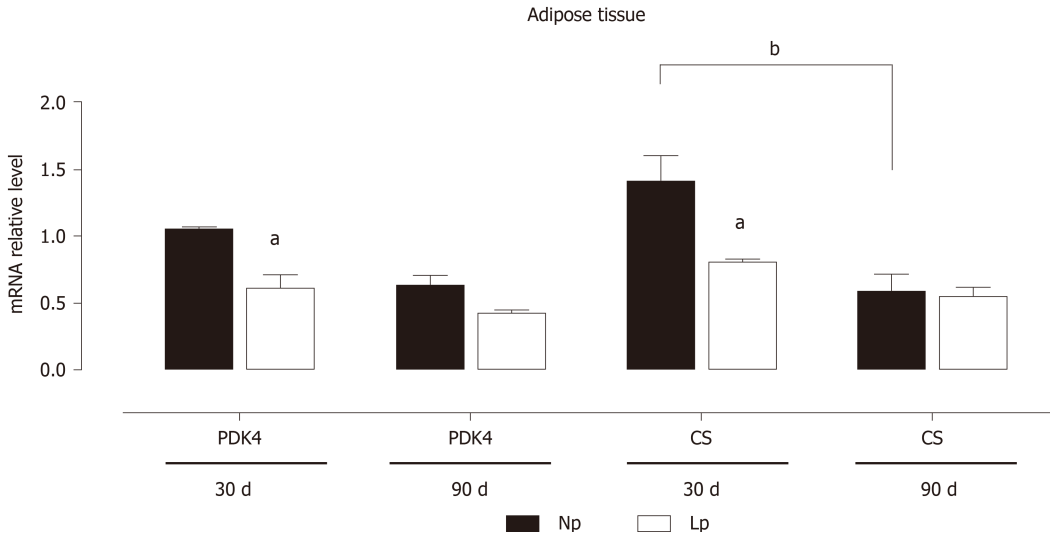

Figure 2 Expression of pyruvate dehydrogenase kinase 4 and citrate synthase mRNA in adipose tissue of rats. Rats were exposed to maternal low protein diet during gestation and lactation, and their RNA extracted from adipose tissue was analyzed by quantitative reverse transcription PCR. Data are shown as mean ± standard error means analyzed by two-way ANOVA with the mother's diet (normal protein; low protein) and age (30 d, 90 d) as variable factors. Bonferroni's post-hoc test was used. Differences between diet groups are indicated by an asterisks; differences between ages are indicated by bars. $^{a}P < 0.05$, $^{b}P < 0.01$. NP: Normal protein; LP: Low protein; PDK4: Pyruvate dehydrogenase kinase 4; CS: Citrate synthase.

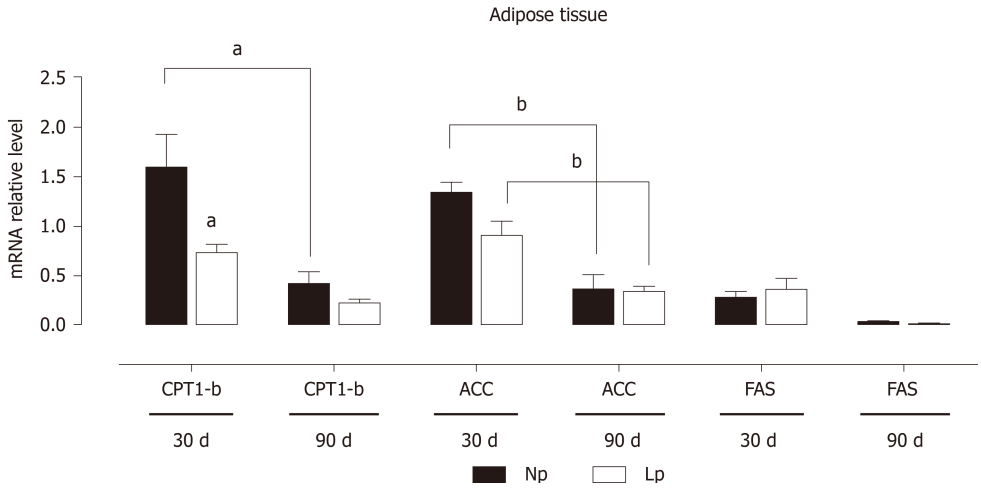

Figure 3 Expression of adipogenic and lipolytic genes in the adipose tissue of rats. Rats were exposed to maternal low protein diet during gestation and lactation, and gene expression of carnitine palmitoylacyltransferase 1, acetyl-CoA carboxylase and fatty acid synthase in adipose tissue was analyzed by quantitative reverse transcription PCR. Data are shown as mean ± standard error means and analyzed by two-way ANOVA with the mother's diet (normal protein; low protein) and age (30 d, 90 d) as variable factors. Bonferroni's post hoc test was used. Differences between diet groups are indicated by an asterisks; differences between ages are indicated by bars. $^{a}P < 0.05$, $^{b}P < 0.01$. NP: Normal protein; LP: Low protein; FAS: Fatty acid synthase; CPT1-b: Carnitine palmitoylacyltransferase 1b; ACC: Acetyl-CoA carboxylase.

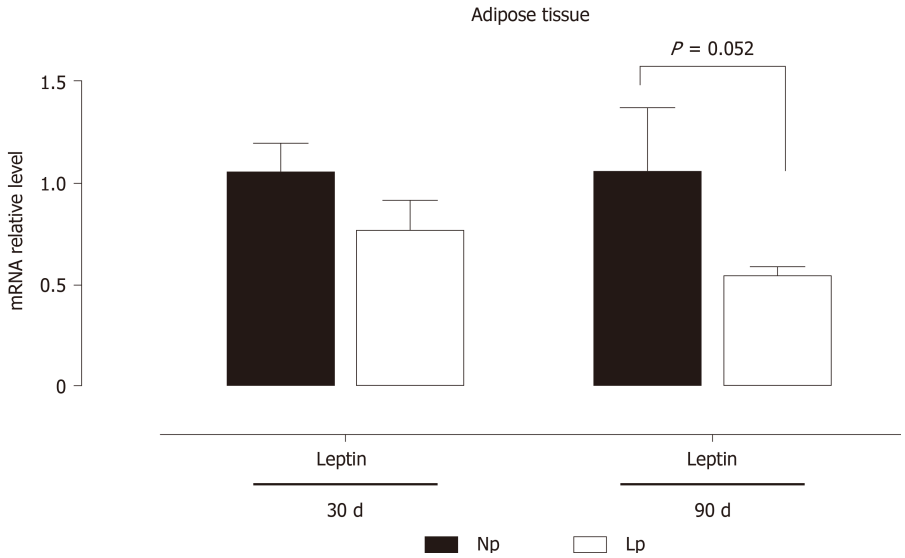

Figure 4 Gene expression of leptin in adipose tissue of rats. Rats were exposed to maternal low protein diet during gestation and lactation, and gene expression of leptin from adipose tissue was analyzed by quantitative reverse transcription PCR. Data are shown as mean ± standard error means and analyzed by two-way ANOVA with the mother's diet (normal protein; low protein) and age (30 d, 90 d) as variable factors. Bonferroni's post hoc test was used. Differences between diet groups are indicated by asterisks. NP: Normal protein; LP: Low protein.

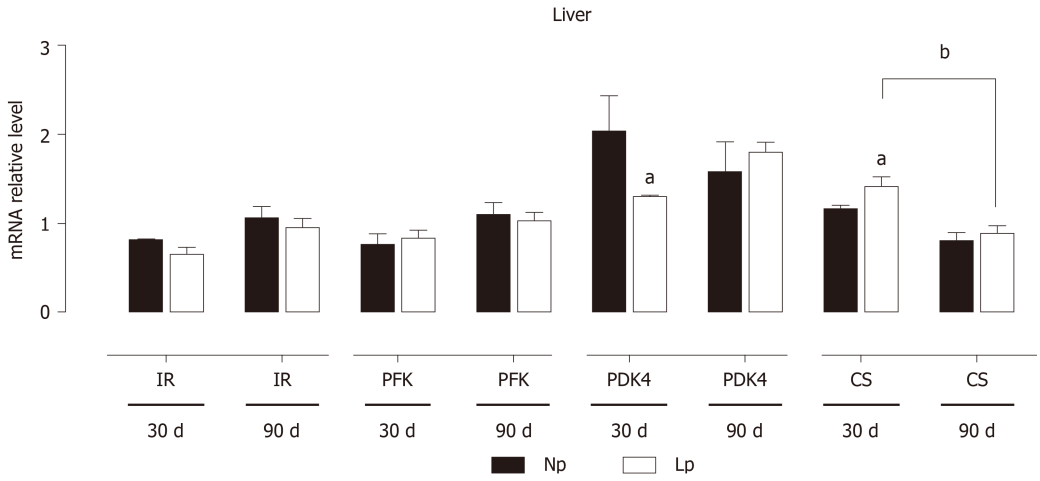

Figure 5 Expression of insulin receptor, glycolytic genes and Krebs cycle genes in the liver of rats. Rats were exposed to maternal low protein diet during gestation and lactation, and gene expression of insulin receptor, phosphofructokinase, pyruvate dehydrogenase kinase 4 and citrate synthase was analyzed by quantitative reverse transcription PCR. Data are shown as mean ± standard error means and analyzed by two-way ANOVA with the mother's diet (normal protein; low protein) and age (30 d, 90 d) as variable factors. Bonferroni's post hoc test was used. Differences between diet groups are indicated by asterisks; differences between ages are indicated by bars. $^{a}P < 0.05$, $^{b}P < 0.01$. NP: Normal protein; LP: Low protein; CS: Citrate synthase; PDK4: Pyruvate dehydrogenase kinase 4; PFK: Phosphofructokinase; IR: Insulin receptor.

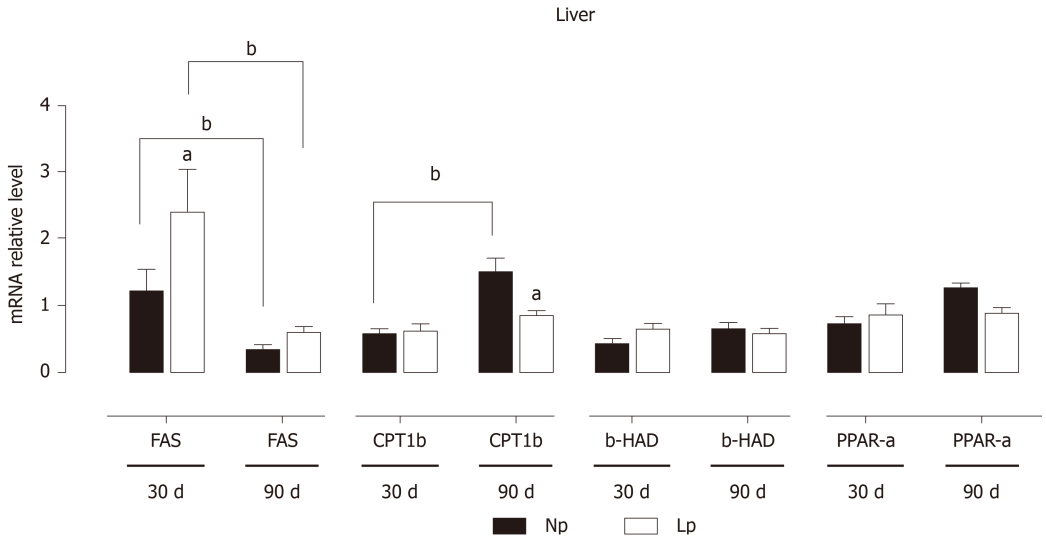

Figure 6 Expression of genes related to lipid metabolism in the liver of rats. Rats were exposed to maternal low protein diet during gestation and lactation, and gene expression was analyzed by quantitative reverse transcription PCR. Data are shown as mean ± standard error means and analyzed by two-way ANOVA with the mother's diet (normal protein; low protein) and age (30 d, 90 d) as variable factors. Bonferroni's post hoc test was used. Differences between diet groups are indicated by asterisks; differences between ages are indicated by bars. $^{a}P < 0.05$, $^{b}P < 0.01$. NP: Normal protein; LP: Low protein; FAS: Fatty acid synthase; CPT1-b: Carnitine palmitoylacyltransferase 1b; b-HAD: Beta hydroxyacyl-coenzyme-A dehydrogenase; PPAR-a: Peroxisome proliferator-activated receptor-alpha.

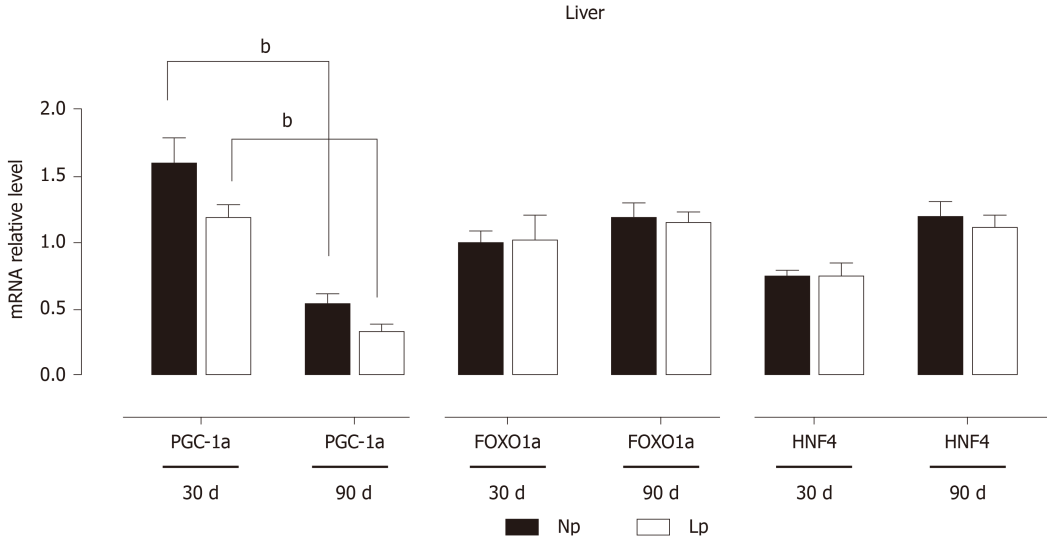

Figure 7 Expression of key transcription factors in the liver of rats. Rats were exposed to maternal low protein diet during gestation and lactation, and gene expression of peroxisome proliferator-activated receptor-alpha coactivator 1 alpha, forkhead box protein O1 and hepatocyte nuclear factor 4 was analyzed by quantitative reverse transcription PCR. Data are shown as mean ± standard error means and analyzed by two-way ANOVA with the mother's diet (normal protein; low protein) and age (30 d, 90 d) as variable factors. Bonferroni's post hoc test was used. Differences between diet groups are indicated by asterisks; differences between ages are indicated by bars. $^{a}P < 0.05$, $^{b}P < 0.01$. NP: Normal protein; LP: Low protein; PGC-1α: Peroxisome proliferator-activated receptor-alpha coactivator 1 alpha; FOXO1a: Forkhead box protein O1; HNF4: Hepatocyte nuclear factor 4.

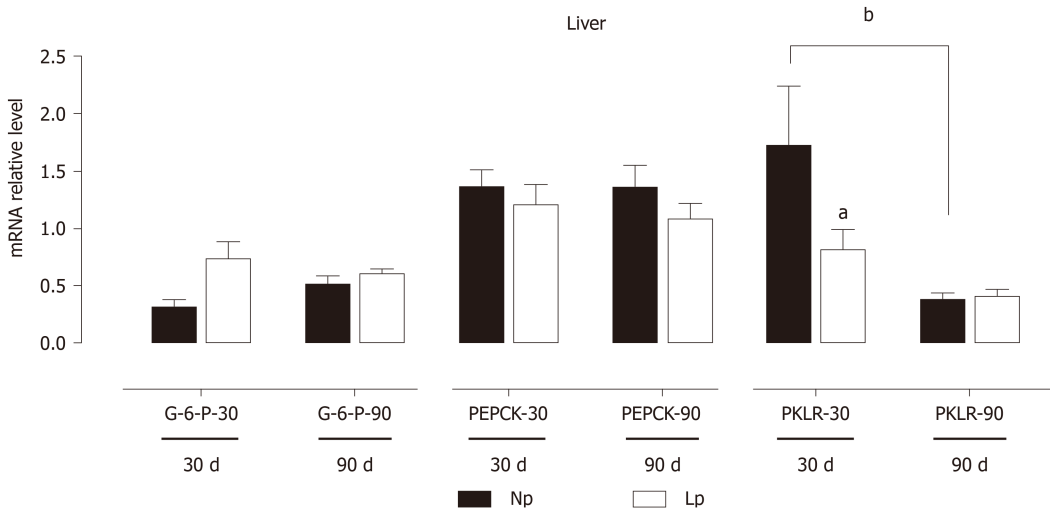

Figure 8 Expression of gluconeogenic genes in the liver of rats. Rats were exposed to maternal low protein diet during gestation and lactation, and gene expression of glucose 6-phosphatase, phosphoenolpyruvate carboxykinase and pyruvate kinase L/R was analyzed by quantitative reverse transcription PCR. Data are shown as mean ± standard error means and analyzed by two-way ANOVA with the mother's diet (normal protein; low protein) and age (30 d, 90 d) as variable factors. Bonferroni's post hoc test was used. Differences between diet groups are indicated by asterisks; differences between ages are indicated by bars. $^{a}P < 0.05$, $^{b}P < 0.01$. NP: Normal protein; LP: Low protein; G6Pase: Glucose 6-phosphatase; PEPCK: Phosphoenolpyruvate carboxykinase; PKLR: Pyruvate kinase L/R.

ARTICLE HIGHLIGHTS

Research background

Perinatal exposure to a poor nutritional environment predisposes the progeny to the development of metabolic disease at the adult age, both in experimental models and humans. Numerous adaptive responses to maternal protein restriction have been reported in metabolic tissues. However, the expression of glucose/fatty acid metabolism-related genes in adipose tissue and liver needs to be described.

Research motivation

To evaluate the metabolic impact of perinatal malnutrition, we determined malnutrition-associated gene expression alterations in liver and adipose tissue.

Research objectives

In the present study, we evaluated the alterations in gene expression of glycolytic/Krebs cycle genes (pyruvate dehydrogenase kinase 4 and citrate synthase), adipogenic and lipolytic genes and leptin in the adipose tissue of offspring rats at 30 d and 90 d of age exposed to maternal isocaloric low protein (LP) diet throughout gestation and lactation. We also evaluated these genes in the livers of the same animals as well as the gene expression of the transcription factors peroxisome proliferator-activated receptor gamma coactivator 1, forkhead box protein O1 and hepatocyte nuclear factor 4 and of gluconeogenic genes.

Research methods

Research methods included animal husbandry, RNA extraction, reverse transcription and quantitative PCR and appropriate statistical analysis.

Research results

In the adipose tissue, we observed a transitory (*i.e.*, at 30 d) downregulation of pyruvate dehydrogenase kinase 4, citrate synthase and carnitine palmitoylacyltransferase 1b gene expression. Such transcriptional changes did not persist in adult LP rats (90 d), but we observed a tendency towards a decreased gene expression of leptin ($P = 0.052$). The liver featured some gene expression alterations comparable to the adipose tissue, such as pyruvate dehydrogenase kinase 4 downregulation at 30 d, and displayed other tissue-specific changes, including citrate synthase and fatty acid synthase upregulation, but pyruvate kinase downregulation at 30 d in the LP group and carnitine palmitoylacyltransferase 1b downregulation at 90 d. These gene alterations, together with previously described changes in gene expression in skeletal muscle, may account for the metabolic adaptations in response to maternal LP diet and highlight the occurrence of persistent transcriptional defects in key metabolic genes that may contribute to the development of metabolic alterations during the adult life as a consequence of perinatal malnutrition.

Research conclusions

We conclude that perinatal malnutrition relays long-lasting transcriptional alterations in metabolically active organs, *i.e.*, the liver and adipose tissue.

Research perspectives

Our observations lay the basis for possible future research directed to human studies.

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ORIGINAL ARTICLE

Observational Study

Evaluation of oxidative stress levels in obesity and diabetes by the free oxygen radical test and free oxygen radical defence assays and correlations with anthropometric and laboratory parameters

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Author contributions: Găman AM, Găman MA and Epîngeac ME designed the study; Epîngeac ME, Găman MA and Găman AM collected the data; Epîngeac ME and Găman AM performed the measurements and contributed reagents; Găman MA analysed the data; Găman MA and Epîngeac ME wrote the paper; Găman AM and Diaconu CC critically revised the paper for scientific content; All authors read and approved the final version of the manuscript; Găman MA and Epîngeac ME contributed equally to this work.

Institutional review board statement: The Ethics Committee of the University of Medicine and Pharmacy of Craiova, Craiova, Romania approved the current study (approval number: 40/27.03.2018).

Informed consent statement: All the subjects involved in the current study agreed to partake in the research and gave their written informed consent. All procedures and experiments were carried out in accordance with the national law and the Helsinki Declaration of 1975, as revised in 2008(5).

Conflict-of-interest statement: The authors have no conflicts of

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Abstract

BACKGROUND

Obesity and diabetes are associated with high levels of oxidative stress. In Romanian patients with obesity and (or) diabetes, this association has not been sufficiently explored.

AIM

To evaluate oxidative stress in obese and (or) diabetic subjects and to investigate the possible correlations between oxidative stress and anthropometric/biochemical parameters.

METHODS

Oxidative stress was evaluated from a single drop of capillary blood. Reactive oxygen species (ROS) were evaluated using the free oxygen radical test (FORT). The free oxygen radical defence (FORD) assay was used to measure antioxidant levels.

RESULTS

FORT levels were higher in obese subjects (3.04 ± 0.36 mmol/L H_2O_2) *vs* controls (2.03 ± 0.14 mmol/L H_2O_2) ($P < 0.0001$). FORD levels were lower in obese subjects

interest to disclose.

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(1.27 ± 0.13 mmol/L Trolox) *vs* controls (1.87 ± 1.20 mmol/L Trolox) (P = 0.0072). Obese diabetic subjects had higher FORT values (3.16 ± 0.39 mmol/L H_2O_2) *vs* non-diabetic counterparts (2.99 ± 0.33 mmol/L H_2O_2) (P = 0.0233). In obese subjects, FORT values correlated positively with body mass index (BMI) (r = 0.48, P = 0.0000), waist circumference (WC) (r = 0.31, P = 0.0018), fasting plasma glucose (FPG) (r = 0.31, P = 0.0017), total cholesterol (TC) (r = 0.27, P = 0.0068) and uric acid (r = 0.36, P = 0.0001). FORD values correlated negatively with BMI (r = -0.43, P = 0.00001), WC (r = -0.28, P = 0.0049), FPG (r = -0.25, P = 0.0130), TC (r = -0.23, P = 0.0198) and uric acid (r = -0.35, P = 0.0002). In obese diabetic subjects, FORT values correlated positively with BMI (r = 0.49, P = 0.0034) and TC (r = 0.54, P = 0.0217). FORD values were negatively associated with BMI (r = -0.54, P = 0.0217) and TC (r = -0.58, P = 0.0121).

CONCLUSION
Oxidative stress levels, as measured by the FORT and FORD assays, were higher in obese subjects *vs* controls. ROS levels were elevated in diabetic obese patients *vs* obese non-diabetic patients and controls.

Key words: Oxidative stress; Obesity; Diabetes; Reactive oxygen species; Antioxidants; Dyslipidaemia

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Core tip: Oxidative stress is involved in obesity, diabetes, and subsequently, diabesity (the co-occurrence of obesity and diabetes). In our study, oxidative stress levels were increased in patients with obesity, diabetes and diabesity. We suggest that the free oxygen radical test and the free oxygen radical defence assays are useful in evaluating the levels of oxidative stress in obesity, diabetes and diabesity. In this study, free oxygen radical test and free oxygen radical defence values also correlated with anthropometric and laboratory parameters in patients with obesity, diabetes and diabesity.

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INTRODUCTION

Worldwide, obesity and type 2 diabetes mellitus (T2DM) have emerged in epidemic proportions. This problematic issue has affected both high- and low-income countries alike, and by 2030 the global prevalence of T2DM is expected to surpass 7.5% (from 6.2% in 2010)[1,2]. According to the country profile released in 2016 by the World Health Organization (WHO), the prevalence of obesity and diabetes in Romania was estimated at 23.4% and 8.4%, respectively[3]. Thus, understanding the molecular mechanisms that drive the development and the evolution of obesity and T2DM are of the utmost importance in our country as well as worldwide.

Both obesity and T2DM are associated with increased levels of oxidative stress. These disorders are characterized by the excessive production of reactive oxygen species (ROS), as well as dysfunctional antioxidant systems[4,5]. Adipose tissue is a veritable endocrine organ, capable of producing adipokines which stimulate the generation of ROS and pro-inflammatory molecules, such as interleukin 1β (IL-1β) and 6 (IL-6) and tumour necrosis factor-alpha (TNF-α)[5]. In T2DM, chronic hyperglycaemia is also a source of ROS, and the ROS-hyperglycaemia crosstalk is involved in the development of the micro- and macro-vascular complications of T2DM[4]. Thus, a vicious cycle in which oxidative stress generates oxidative stress commences. In this context, the increased levels of oxidative stress in obese and diabetic subjects might explain the development of diabesity, *i.e.*, the occurrence of T2DM in obese individuals[2].

Due to the limited knowledge regarding the crosstalk between obesity, T2DM and

oxidative stress in Romanian patients, in the current study, we aimed to evaluate oxidative stress levels in obese non-diabetic and obese diabetic subjects, and to identify possible correlations between biochemical and oxidative stress parameters in these patients.

MATERIALS AND METHODS

Study subjects

A total of 102 obese subjects were recruited from several outpatient clinics in Craiova, a city in southwest Romania, for inclusion in the study group. The patients were classified as obese and stratified into obesity classes in accordance with the WHO definition of obesity: Body mass index (BMI) > 30 kg/m^2[6]. T2DM was diagnosed based on the American Diabetes Association criteria[7]. Thirty healthy individuals were selected for the control group.

Oxidative stress assessment

Oxidative stress was evaluated from a single drop of capillary blood using the CR3000 analyser (Callegari, the Catellani group, Parma, Italy). The CR3000 analyser uses two colorimetric assays to evaluate oxidative stress: The free oxygen radical test (FORT) and the free oxygen radical defence (FORD). The FORT reflects the levels of ROS in the blood, and in normal individuals should have a value ≤ 2.3 mmol/L H_2O_2. The FORD assay reflects the levels of antioxidants in the blood, and in normal individuals has a value in the 1.07-1.53 mmol/L Trolox range. Both FORT and FORD are valuable tests in assessing oxidative stress levels in patients with T2DM and obesity and have been used in research for more than 10 years. The detailed principles of the assays are described elsewhere[8]. Reagents were also purchased from Callegari, the Catellani group, Parma, Italy.

Assessment of demographic, clinical and biochemical parameters

The following demographic and clinical parameters were evaluated: Age, sex, weight and height to calculate the BMI and waist circumference (WC). The following laboratory variables were evaluated by standard methods: Fasting plasma glucose (FPG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG) and uric acid (UA). The estimated glomerular filtration rate (eGFR) was calculated using the MDRD formula.

Statistical analysis

Categorical variables are reported as frequencies and percentages and continuous variables as the mean ± SD. Categorical variables were compared using Fisher's exact test. Continuous variables were compared using independent samples *t*-test. All variables were tested to check the normal distribution of the data. The Pearson and the Spearman correlation coefficients were employed for parametric and nonparametric variables to investigate the possible associations between FORT or FORD and other biochemical parameters. The level of significance was presented as *P* values and the analysis was performed at the 5% level of significance using GraphPad QuickCalcs (https://www.graphpad.com), MedCalc (https://www.medcalc.org) and Microsoft Excel (Microsoft Office Professional Plus 2013).

RESULTS

The demographic, clinical and laboratory parameters of the study population are reported in Table 1. In this study, we included 102 obese patients (mean age: 62.14 ± 10.19 years, 79.41% female) and 30 healthy controls (mean age: 44.60 ± 18.76 years, 70.00% female). Obese patients had higher BMI (35.75 ± 3.75 kg/m^2 *vs* 24.56 ± 1.78 kg/m^2, $P < 0.0001$), WC (106.58 ± 13.27 cm *vs* 96.38 ± 3.76 cm, $P < 0.0001$), FPG (117.56 ± 42.13 mg/dL *vs* 92.63 ± 7.60 mg/dL, $P = 0.0016$), TC (228.66 ± 45.22 mg/dL *vs* 137.70 ± 20.46 mg/dL, $P < 0.0001$), LDL-c (141.78 ± 42.43 mg/dL *vs* 106.93 ± 15.93 mg/dL, $P < 0.0001$), TG (156.98 ± 92.25 mg/dL *vs* 95.77 ± 20.22 mg/dL, $P = 0.0005$), UA (4.44 ± 1.18 mg/dL *vs* 3.80 ± 0.92 mg/dL, $P = 0.0073$), and lower HDL-c (47.72 ± 12.68 mg/dL *vs* 66.23 ± 10.85 mg/dL, $P < 0.0001$) and eGFR (68.49 ± 18.84 mL/min/1.73 m^2*vs* 106.77 ± 17.75 mL/min/1.73 m^2, $P < 0.0001$) *vs* controls. FORT values were higher (3.04 ± 0.36 mmol/L H_2O_2 *vs* 2.03 ± 0.14 mmol/L H_2O_2, $P < 0.0001$) and FORD levels were lower (1.27 ± 0.13 mmol/L Trolox *vs* 1.87 ± 1.20 mmol/L Trolox, $P = 0.0072$) in obese patients as compared to healthy controls.

The study population included 33 (32.35%) obese patients diagnosed with T2DM

Table 1 Demographic, clinical and biochemical parameters of the study population *vs* controls

	Obese subjects	Controls	*P* value
Male/Female (*n*)	21/81	9/21	0.3234
Age (yr)	62.14 (10.19)	44.60 (18.76)	< 0.0001
BMI (kg/m^2)	35.75 (3.75)	24.56 (1.78)	< 0.0001
WC (cm)	106.58 (13.27)	96.38 (3.76)	< 0.0001
FPG (mg/dL)	117.56 (42.13)	92.63 (7.60)	0.0016
TC (mg/dL)	228.66 (45.22)	137.70 (20.46)	< 0.0001
HDL-c (mg/dL)	47.72 (12.68)	66.23 (10.85)	< 0.0001
LDL-c (mg/dL)	141.78 (42.43)	106.93 (15.93)	< 0.0001
TG (mg/dL)	156.98 (92.25)	95.77 (20.22)	0.0005
UA (mg/dL)	4.44 (1.18)	3.80 (0.92)	0.0073
eGFR (mL/min/1.73 m^2)	68.49 (18.84)	106.77 (17.75)	< 0.0001
FORT (mmol/L H_2O_2)	3.04 (0.36)	2.03(0.14)	< 0.0001
FORD (mmol/L Trolox)	1.27 (0.13)	1.87 (1.20)	0.0072

Data are presented as mean ± SD or as *n*. BMI: Body mass index; WC: Waist circumference; FGP: Fasting plasma glucose; TC: Total cholesterol; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; TG: Triglycerides; UA: Uric acid; eGFR: Estimated glomerular filtration rate; FORT: Free oxygen radical test; FORD: Free oxygen radical defence.

and 69 (67.65%) obese patients without T2DM. The demographic, clinical and laboratory parameters of the obese T2DM subjects *vs* the obese non-diabetic patients are shown in Table 2.

Obese diabetic subjects were older (66.45 ± 9.78 years *vs* 60.07 ± 9.79 years, P = 0.0027), had higher FPG (153.32 ± 56.94 mg/dL *vs* 100.45 ± 13.91 mg/dL, P < 0.0001) and lower HDL-c (43.98 ± 10.48 mg/dL *vs* 49.51 ± 13.30 mg/dL, P = 0.0384) *vs* obese subjects without diabetes. Also, obese subjects with T2DM recorded higher FORT values (3.16 ± 0.39 mmol/L H_2O_2 *vs* 2.99 ± 0.33 mmol/L H_2O_2, P = 0.0233) *vs* obese subjects without diabetes.

In obese subjects, we recorded positive correlations between FORT and BMI (r = 0.48, P = 0.0000), WC (r = 0.31, P = 0.0018), FPG (r = 0.31, P = 0.0017), TC (r = 0.27, P = 0.0068) and UA (r = 0.36, P = 0.0001). Also, in obese subjects, FORD correlated negatively with the BMI (r = -0.43, P = 0.00001) (Figure 1), WC (r = -0.28, P = 0.0049), FPG (r = -0.25, P = 0.0130), TC (r = -0.23, P = 0.0198) and UA (r = -0.35, P = 0.0002).

In obese diabetic subjects, we detected strong positive associations between FORT and BMI (r = 0.49, P = 0.0034) (Figure 2), and FORT and TC (r = 0.54, P = 0.0217). Also, FORD was negatively associated with BMI (r = -0.54, P = 0.0217) and TC (r = -0.58, P = 0.0121).

DISCUSSION

Our study focussed on the evaluation of oxidative stress in obese (diabetic and non-diabetic) patients, and showed that ROS levels (assessed by the FORT assay) are increased and antioxidant levels (assessed by the FORD assay) are decreased in patients diagnosed with obesity *vs* healthy controls. Moreover, obese patients who also had T2DM had higher ROS levels *vs* obese non-diabetic subjects and healthy controls. In obese subjects with T2DM, FORD levels were not significantly decreased as compared to non-diabetic obese patients; thus, we may assume that, in T2DM, the body might produce supplementary amounts of antioxidants to scavenge the excessive amount of free oxygen radicals (Figure 3).

ROS levels are increased in obesity and diabesity

Our study reinforced that ROS levels are increased in subjects with obesity and that the elevation in ROS is more pronounced in obese patients who have T2DM. Moreover, in obese patients, we detected positive correlations between FORT, which measures ROS levels in the body, and BMI, WC, FPG, TC and UA. Also, in patients diagnosed with diabesity (the association between obesity and T2DM), we recorded higher FORT values *vs* subjects with obesity and healthy controls. Similarly, Pavlatou *et al*[8] also evaluated oxidative stress levels in T2DM subjects using the FORT and FORD assays, and reported increased FORT values in diabetic patients *vs* healthy

Table 2 Demographic, clinical and biochemical parameters of obese type 2 diabetes mellitus subjects *vs* obese non-diabetic patients

	T2DM	Obese non-diabetic subjects	*P* value
Male/Female (*n*)	6/27	15/54	0.7965
Age (yr)	66.45 (9.78)	60.07 (9.79)	0.0027
BMI (kg/m^2)	36.66 (4.01)	35.32 (3.57)	0.0920
WC (cm)	107.24 (13.91)	106.26 (13.04)	0.7286
FPG (mg/dL)	153.32 (56.94)	100.45 (13.91)	< 0.0001
TC (mg/dL)	231.92 (46.75)	227.11 (44.74)	0.6180
HDL-c (mg/dL)	43.98 (10.48)	49.51 (13.30)	0.0384
LDL-c (mg/dL)	137.54 (38.10)	143.80 (44.47)	0.4885
TG (mg/dL)	149.41 (111.44)	160.60 (82.18)	0.5690
UA (mg/dL)	4.70 (1.33)	4.32 (1.10)	0.1319
FORT (mmol/L H_2O_2)	3.16 (0.39)	2.99 (0.33)	0.0233
FORD (mmol/L Trolox)	0.67 (0.15)	0.72 (0.14)	0.0649

Data are presented as mean ± SD or as *n*. T2DM: Type 2 diabetes mellitus; BMI: Body mass index; WC: Waist circumference; FGP: Fasting plasma glucose; TC: Total cholesterol; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; TG: Triglycerides; UA: Uric acid; eGFR: Estimated glomerular filtration rate; FORT: Free oxygen radical test; FORD: Free oxygen radical defence.

controls. Furthermore, their study demonstrated positive correlations between FORT and BMI, WC, LDL-c and TG. However, in T2DM patients, we found positive correlations between FORT and BMI (r = 0.49, P = 0.0034). Positive correlations between FORT and WC (r = 0.31, P = 0.0018) were only seen in obese non-diabetic subjects in our research. Thus, our data support the hypothesis that excessive body weight is associated with increased levels of oxidative stress, as ROS values increased and antioxidant levels decreased with increased BMI. Obesity is characterized by redox alterations induced and linked with excessive dietary intake, chronic fat cell inflammation, mitochondrial dysfunction, glycoxidation and oxidation of fatty acids. Moreover, oxidative stress seems to be related to the development of insulin resistance in T2DM. Obese subjects have insulin resistance which, in turn, causes compensatory hyperinsulinaemia and can explain the development of diabesity[9,10].

Antioxidant deficiency in obesity and diabesity

In our study, we detected decreased FORD values in obese patients. Also, FORD, which reflects the levels of antioxidants in the body, was negatively correlated with BMI (r = -0.54, P = 0.0217). Surprisingly, the difference in FORD levels between patients with obesity and those with diabesity was rather unremarkable. Thus, we may assume that, in T2DM, the body is forced to produce larger amounts of endogenous antioxidants to counteract the increase in ROS. Similar to our findings, Pavlatou *et al*[8] also reported decreased FORD levels in patients diagnosed with T2DM. However, in their research, the mean BMI of the patients was 29.3 ± 5.7 kg/m^2 as opposed to 28.7 ± 4.2 kg/m^2 in controls (P = 0.62). Thus, T2DM patients included in the aforementioned study were mostly overweight or were diagnosed with class I obesity. Despite the excessive generation of oxidative stress in obesity and T2DM, antioxidant supplementation remains controversial. Current evidence recommends lifestyle changes as a first step in the management of these disorders, with physical exercise and low-calorie, antioxidant-rich diets as key elements of the therapeutic armamentarium[11].

The oxidative stress-dyslipidaemia crosstalk

In the present study, TC correlated positively with FORT and TC (r = 0.27, P = 0.0068) and negatively with FORD (r = -0.23, P = 0.0198) in obese patients. Moreover, TC also correlated positively with FORT (r = 0.54, P = 0.0217) and negatively with FORD (r = -0.58, P = 0.0121) in diabetic obese patients. However, Pavlatou *et al*[8] reported a positive correlation between LDL-c and FORT (r = 0.03, P = 0.05). The FORT-TC and FORD-TC correlations reported in our study might be explained by the accumulation of toxic lipids which lead to lipotoxicity in diabetic and (or) obese patients[10]. In T2DM, dyslipidaemia is highly prevalent, with nearly 70% of patients having high TG and LDL-c values and low HDL-c[12]. Hypercholesterolaemia and obesity are recognized risk factors in the development of T2DM, but adherence to lipid-lowering drugs in patients with T2DM remains low[13-15]. HDL-c is a major candidate in the

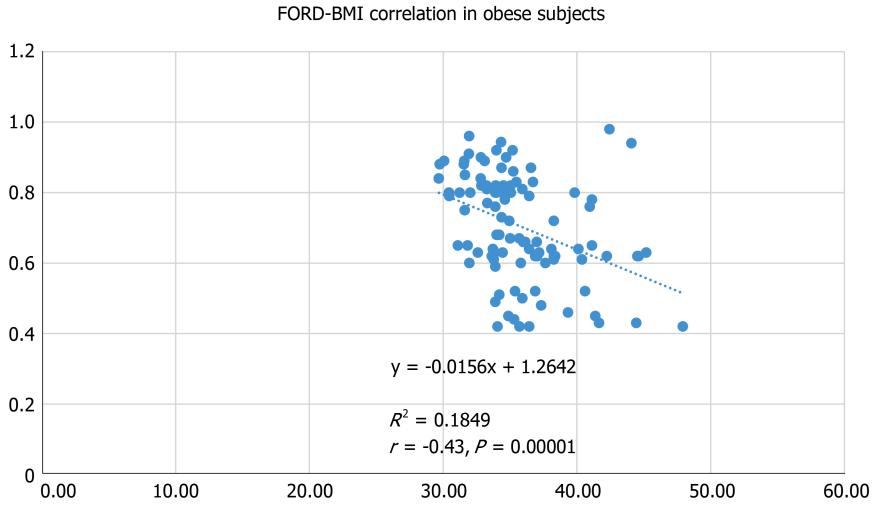

Figure 1 Correlation between free oxygen radical defence values and body mass index in obese patients.
FORD: Free oxygen radical defence; BMI: Body mass index.

antioxidant defence against ROS-induced damage, and its myriad of positive effects in health (anti-inflammatory, antioxidant, anti-atherogenic *etc*.) are related to its strong cooperation with antioxidant enzymes, such as superoxide dismutase (SOD) or paraoxonase-1 (PON-1). The activity of PON-1 and SOD is reduced in obese dyslipidaemic patients[5]. Moreover, studies have shown that the activity of antioxidant enzymes, such as SOD or catalase, is also decreased in T2DM. It seems that the risk of developing T2DM is higher amongst catalase-deficient subjects[4]. On the other hand, Picu *et al*[16] reported only slightly lower SOD and total antioxidant status values, and slightly higher UA levels in T2DM patients as compared to controls. However, they did report a higher total oxidant status in T2DM patients *vs* healthy controls. Increased levels of UA might be related to development of the metabolic syndrome. In the metabolic syndrome, UA has been shown to stimulate the generation of ROS by fat cells and stimulates lipid peroxidation[17]. The role of oxidative stress in T2DM is complex, and experimental studies have concluded that chronic oxidative stress levels in pancreatic beta cells lead, *via* chronic hyperglycaemia-caused glucotoxicity, to a loss of expression of the endogenous insulin gene[18]. In addition, the contribution of high ROS levels, low antioxidant defences and lipid abnormalities in carcinogenesis should not be forgotten[19-21]. We previously reported that diffuse large B-cell lymphoma is associated with increased ROS levels and low HDL-c and antioxidant values[22]. Moreover, low HDL-c levels have also been linked to other types of non-Hodgkin's lymphoma, breast, lung, gynaecological or prostate cancers, as well as other malignancies[23].

Strengths and limitations

Our research has some strengths. Firstly, the involvement of oxidative stress in T2DM and (or) obesity in the population from southwest Romania has received little attention in studies. We believe this is the first report to evaluate oxidative stress in obese and (or) diabetic patients using the FORT and FORD assays in Romania. Moreover, we evaluated ROS and antioxidant levels using a point-of-care method which has been employed in research for over ten years, and we reported not only that oxidative stress levels are increased in obese and (or) diabetic subjects, but also the correlations between FORT or FORD and anthropometric/biochemical parameters. The current study confirms our previous research findings, *i.e*., that obesity is associated with increased oxidative stress levels[24]. However, our research initiative has several limitations. We included a relatively small number of patients and we were unable to recruit a control group of diabetic non-obese subjects. In addition, the CR3000 was not designed to evaluate urinary oxidative stress parameters. We will work on addressing these limitations in the near future.

In conclusion, oxidative stress levels, as measured by the FORT and FORD assays, were higher in obese subjects *vs* healthy controls. ROS levels were elevated in diabetic obese patients *vs* obese non-diabetic patients and healthy controls. Obese patients had higher BMI, WC, FPG, TC, LDL-c, TG, UA and lower HDL-c *vs* healthy controls. In obese subjects, FORT levels correlated positively with BMI, FPG, TC and UA, and FORD levels correlated negatively with BMI, WC, FPG, TC and UA. Obese diabetic subjects were older, had higher FPG and lower HDL-c. In obese diabetic subjects,

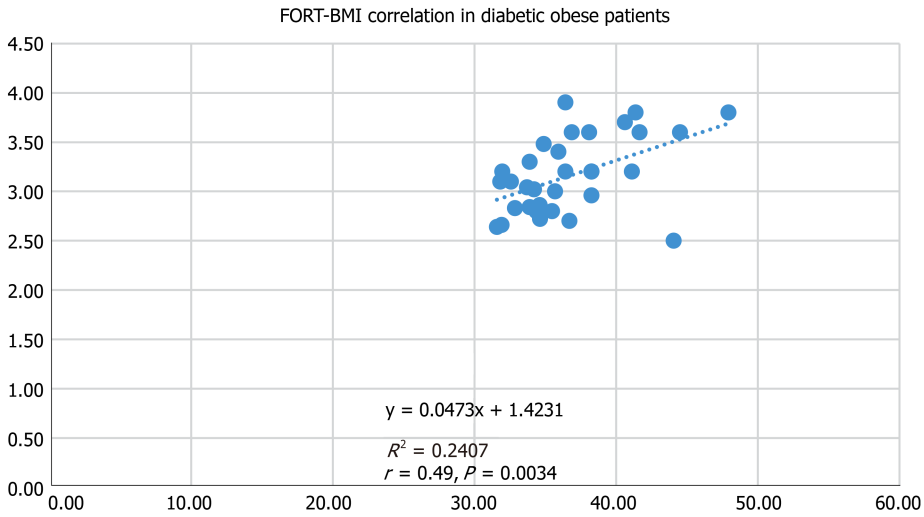

Figure 2 Correlation between free oxygen radical test values and body mass index in diabetic obese patients. FORT: Free oxygen radical test; BMI: Body mass index.

FORT levels correlated positively with BMI and TC, and FORD levels correlated negatively with BMI and TC. Taken together, these findings indicate that the management of obesity and (or) diabetes should also take into consideration strategies to reduce ROS levels and increase the antioxidant capacity of the body, in addition to the treatment of lipid abnormalities. However, further studies are needed to clarify the crosstalk between oxidative stress, obesity and diabesity.

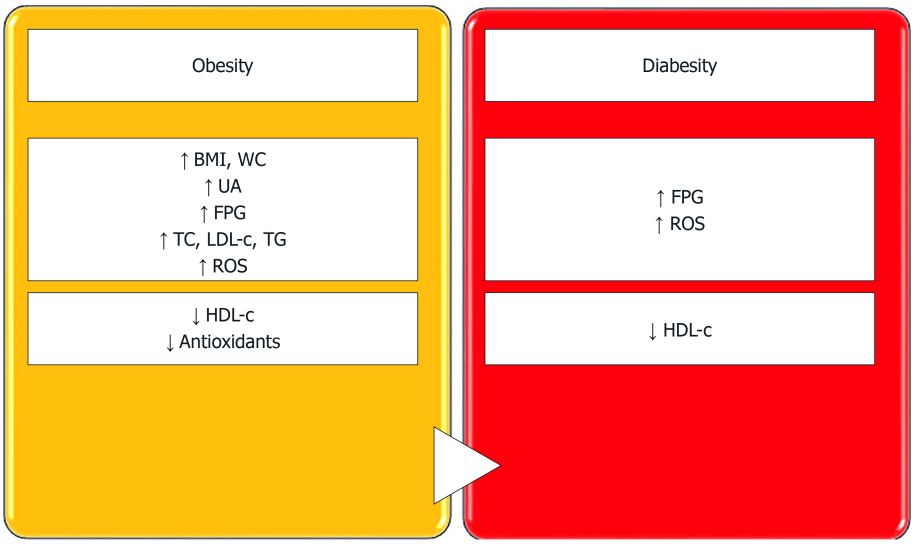

Figure 3 The key findings in our study regarding the changes in oxidative stress levels and other clinical and (or) biochemical parameters in patients with obesity and diabesity. BMI: Body mass index; WC: Waist circumference; UA: Uric acid; FGP: Fasting plasma glucose; TC: Total cholesterol; LDL-c: Low-density lipoprotein cholesterol; TG: Triglycerides; ROS: Reactive oxygen species; HDL-c: High-density lipoprotein cholesterol.

ARTICLE HIGHLIGHTS

Research background

Oxidative stress is a key player in health and disease, and its particular involvement in the development of obesity, type 2 diabetes mellitus, cardiovascular disorders, neurodegeneration and cancer have attracted much attention from the scientific community in recent years.

Research motivation

The motivation for our research was to contribute to the study of oxidative stress involvement in obesity, diabetes and their co-occurrence (diabesity), and to improve the current knowledge regarding the development of these public health problems.

Research objectives

The main objectives of this study were to evaluate oxidative stress levels in obesity, diabetes and diabesity using the free oxygen radical test (FORT) and the free oxygen radical defence (FORD) tests. In addition, we investigated whether FORT and (or) FORD values correlated with anthropometric and laboratory parameters.

Research methods

Oxidative stress was evaluated from a single drop of capillary blood using the CR3000 analyser by two colorimetric assays: The free oxygen radical test (FORT) and the free oxygen radical defence (FORD) assays. Demographic, clinical and biochemical parameters were assessed by standard methods.

Research results

FORT levels were higher in obese subjects *vs* healthy controls and correlated positively with body mass index, waist circumference, fasting plasma glucose, total cholesterol and uric acid. FORD levels were lower in obese subjects *vs* healthy controls and correlated negatively with body mass index, waist circumference, fasting plasma glucose, total cholesterol and uric acid. Patients with diabesity had higher FORT values *vs* non-diabetic counterparts. In these subjects, FORT levels correlated positively with body mass index and total cholesterol, and FORD levels was negatively associated with body mass index and total cholesterol.

Research conclusions

Oxidative stress levels are increased in obese subjects. In patients with diabesity, reactive oxygen species are elevated *vs* obese non-diabetic subjects and controls.

Research perspectives

Further studies are needed to clarify the role of oxidative stress in obesity, diabetes and diabesity, and to transpose these results from bench to bedside. The value of antioxidants in the management of these public health problems needs further clarification.

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ORIGINAL ARTICLE

Prospective Study

Severity of the metabolic syndrome as a predictor of prediabetes and type 2 diabetes in first degree relatives of type 2 diabetic patients: A 15-year prospective cohort study

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Author contributions: Amini M generated the idea and designed the Isfahan diabetes prevention study, participated in most of the experiments, revising the paper critically for important intellectual content; Meamar R generated the idea and designed the current secondary study, wrote all major parts of the article and checked it for intellectual content and revised it; Feizi A supervised the current secondary study and drafted the paper and revised it critically for important intellectual content and performed all statistical analyses; Aminorroaya A essentially participated as co-investigator in Isfahan diabetes prevention study, revised paper critically for important intellectual content; Nasri M edited and resolved grammatical errors in the article; Abyar M assisted in data collection and preparation and statistical analysis.

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Abstract

BACKGROUND

Type 2 diabetes mellitus (T2DM) has high morbidity and mortality worldwide, therefore there is of paramount importance to identify the risk factors in the populations at risk early in the course of illness. A strong correlation between severity of metabolic syndrome (MetS) and HbA1c, fasting insulin and insulin resistance has been reported. Accordingly, the MetS severity score (or MestS Z-score) can potentially be used to predict the risk of T2DM progression over time.

AIM

To evaluate the association the of MestS Z-score in first degree relatives (FDRs) of T2DM with the risk of prediabetes and type 2 diabetes in future.

METHODS

A prospective open cohort study was conducted between 2003-2018. At baseline, the sample comprised of 1766 FDRs of patients with T2DM who had a normal glucose tolerance test. Relative risk (RR) and 95% confidence interval were calculated based on logistic regression. The receiver-operator characteristic analysis and area under the curve based on MetS Z-score were used to evaluate the risk of prediabetes and diabetes among the FDR population.

RESULTS

Baseline MetS Z-scores were associated with the its latest values ($P < 0.0001$).

Sciences, Isfahan, Iran. Study project number: 95017.

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Compared with individuals who were T2DM free at the end of follow up, those who developed T2DM had higher MetS Z-score at baseline ($P < 0.001$). In multivariable logistic regression analyses for every unit elevation in MetS Z-score at the baseline, the RR for developing future T2DM and prediabetes was (RR = 1.94, RR = 3.84), (RR = 1.5, RR = 2.17) in total population and female group, respectively ($P < 0.05$). The associations remained significant after adjusting the potential confounding variables. A cut off value of 0.97 and 0.94 was defined in the receiver-operator characteristic curve based on the MetS Z-score for differentiating female patients with diabetes and prediabetes from the normal population, respectively.

CONCLUSION
The MetS Z-score was associated with an increased risk of future T2DM. Appropriate interventions at earlier stages for preventing and attenuating MetS effects may be considered as an effective strategy for FDR as at-risk population.

Key words: Insulin resistance; Metabolic syndrome; Risk; Type 2 diabetes mellitus; Prediabetes; First degree relative

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Core tip: This prospective cohort study showed that metabolic syndrome severity score at baseline, in first degree relative of type 2 diabetes mellitus patients with normal glucose tolerance, predicts the incidence of future diabetes and prediabetes. In this study, the cut off values of metabolic syndrome Z-score for predicting prediabetes and diabetes were 0.94 and 0.97, respectively. This negligible difference between two groups in terms of cut off values highlights the importance of intervention at prediabetes stage.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) has high morbidity and mortality worldwide, therefore there is of paramount importance to identify risk factors in the populations at risk early in the course of illness[1].

One of the important factors in increasing the risk of acquiring DM is metabolic syndrome (MetS). MetS consists of having three of the five following abnormalities; central obesity, hypertension, high triacylglycerol, low high-density lipoproteins (HDL) cholesterol and elevated fasting glucose[2]. MetS is linked to insulin resistance[3] and obesity[4] due to abnormality in cellular function[5]. MetS is a risk factor for future cardiovascular disease (CVD)[6,7] and T2DM[7-9] both in children and adults[10,11]. Linear measurements of MetS Z-score is valuable not only for the identification of high-risk individuals but also for following up the disease development over time and evaluation of the response to treatment[12]. Recently the potential benefit of measuring MetS Z-score in childhood and predicting cardiovascular disease and diabetes later in life has been revealed[12-13].

The MetS not only is inherited but also influenced by lifestyle and genetic factors[14]. This provides an opportunity to modify lifestyle or intervene with treatments in the population who have been recognized to be at risk for prediabetes and diabetes mellitus such as the offspring of the patients with T2DM[15].

First degree relatives (FDRs) with T2DM are at higher risk of diabetes and pre-diabetes progression[16-18].

A study by Siewert *et al*[19] indicated that the prevalence of MetS is high in young FDR adults. Iran is a developing country and its population is adopting a more sedentary lifestyle with new diets resulting in a high prevalence of T2DM and prediabetes[20]. In this study, taking into account potential links between MetS Z-score

as a marker for prediabetes or T2DM risk, we assessed the role of MetS Z-score for predicting prediabetes and T2DM in FDR population in a long-term follow-up cohort study to enable clinicians to identify and treat this high-risk population through conducting of interventions for preventing MetS or diminishing its side effects.

MATERIALS AND METHODS

Study design and population

Data were drawn from the database of the Isfahan Diabetes Study on the first degree relatives (FDR), the details of the study have been presented elsewhere[21]. In summary, the study is an ongoing open cohort study started at 2003 on FDR of patients with T2DM in Isfahan, a large city in central Iran, to measure several possible risk factors of diabetes incidence. At baseline, our sample comprised of 3492 FDRs of T2DM patients. All participants were visited at the Isfahan Endocrine and Metabolism Research Center, affiliated to Isfahan University of Medical Sciences, Iran. The Bioethics Committee of Isfahan University of Medical Sciences approved the study and written informed consent was obtained from every participant based on the Declaration of Helsinki.

At the time of examination, subjects underwent anthropometric measurements and laboratory tests, including a standard 75-g 2-h oral glucose tolerance test (OGTT). For the current study, the analysis was limited to those normal glucose test (NGT) participants without missing data *i.e*. 1766 at baseline. NGT participants were followed from 2003 annually until 2018 and then classified to NGT, impaired glucose test and T2DM according to the American Diabetes Association criteria[22].

Variables assessment

Anthropometric and demographic variables: All participants completed a demographic questionnaire including age, gender, level of education, smoking and personal and medical history at baseline. Anthropometric and basic clinical measurements, including body mass index (kg/m^2), waist circumference (WC, cm) and waist-to-hip ratio (WHR) were calculated according to standard methods[23] and blood pressure (BP) including both systolic and diastolic were recorded.

MetS Z-Score calculation: Traditional MetS was defined using the National Cholesterol Education Program Adult Treatment Panel-III criteria[2]. Participants had to meet three or more of the following five criteria: (1) Concentration of triacylglycerol ≥ 1.69 mmol/L (150 mg/dL); (2) HDL-cholesterol level < 1.04 mmol/L (40 mg/dL) for men and < 1.3 mmol/L (50 mg/dL) for women; (3) WC ≥ 102 cm for men and 88 cm for women; (4) Glucose concentration ≥ 5.55 mmol/L (100 mg/dL); and (5) systolic BP ≥ 130 mmHg or diastolic BP ≥ 85 mmHg. The MetS Z-score was calculated for adolescents at first visit using formulas published elsewhere[24]. Data collection was conducted at baseline and at follow-up according to the standards of Medical Care in Diabetes[25].

Laboratory parameters: All participants received a 75-g OGTT following a 12- hour overnight fasting period. Plasma glucose (PG) was measured at 0, 30, 60 and 120 min[26] (2-h PG) and fasting plasma glucose (FPG) (mg/dL) was measured by Pars Azmon kit Lot number: 94011 (a photometric method). HbA1c, cholesterol (LDL, HDL) and triglyceride were also measured.

Participants with FPG ≥ 200 mg/dL were considered diabetic. If FPG was ≥ 126 and < 200 mg/dL, a second FPG was measured on another day. If the second FPG was also ≥ 126 mg/dL, participants were classified as diabetic. Those with FPG ≥ 126 mg/dL or 2-h PG ≥ 200 mg/dL were also defined as diabetic. Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) are intermediate stages in the natural history of type 2 diabetes and are called the pre-diabetes phase[22]. FPG < 126 mg/dL with a 2-h PG concentration ≥ 140 and < 200 mg/dL was interpreted as IGT. If FPG was in the range of 100–126 mg/dL and 2-h PG was < 140 mg/dL, it was considered as IFG. NGT was defined as FPG below 100 mg/dL and 2-h PG less than 140 mg/dL[27].

Statistical analysis

Quantitative variables are presented as mean ± SD or median [IQR], while qualitative as frequency (percentage). Depending on the normal or non-normal distribution of data, independent samples *t*-test or Mann-Whitney *U* tests were used for comparing continuous data between NPG and diabetes or prediabetes. Categorical data were compared with the χ^2 test.

Pearson correlation coefficient was calculated for evaluating the correlation of MetS

Z-score at first and last visit. The diagnostic accuracy of MetS Z-Score was evaluated by using the receiver operating characteristic (ROC) curve analysis and calculated area under the curve (AUC) and 95% confidence interval (CI) for AUC. We used binary logistic regression analysis for evaluating the predictive value of MetS Z-Score for diabetes and prediabetes incidence in the future in different models. In these analyses, after obtaining relative risk (RR) and 95%CI in the crude model, the adjustment was made for age and gender in the model 1. All statistical calculations were carried out with the SPSS 15 for Windows (SPSS Inc., Chicago, IL, United States) and $P < 0.05$ was used as a statistically significant level.

RESULTS

Over the fifteen years follow-up from the 1766 NGT participants at baseline, 78 participants developed DM (7.4%), 255 participants progressed to IFG (24.1%) and 89 participants developed IGT (8.4%). Overall, 344 (32.6%) developed pre-diabetes and 630 (59.7%) participants remained NGT. Of this total, 714 were missed to follow up due to moving geographically, withdrawing consent or changing contact details and being unavailable.

Table 1 presents the anthropometric, laboratory and clinical characteristics at the beginning of the study for those participants who developed to diabetes and those who remained normal at follow up periods. All glycemic variables including PG in 0, 30, 60 and 120 min, HbA1c, FPG, and total cholesterol and triglyceride as well as WHR were significantly different between diabetes and a normal group, particularly in total population and female group.

Table 2 presents the anthropometric measurements, laboratory and clinical characteristics at the beginning of the study for those participants who developed to pre-diabetes and those who remained normal. It illustrates a significant difference in glucose level at 0, 30, 60,120 min and triglyceride level, WHR and both systolic and diastolic blood pressure measurement in total population and female group when comparing pre-diabetes group with the normal group. There was a highly significant correlation in terms of MetS Z-score at first and last visits in the FDR population ($r = 0.67$; $P < 0.001$) (Figure 1).

Figure 2 provides MetS Z-score at the first and final visits by diabetes disease status for two groups: Those who did not have diabetes at any of the two visits and those who developed T2DM between first and last visits.

Table 3 presents the results of crude and multivariable binary logistic regression analysis in different models for the association between MetS Z-score levels at baseline and diabetes and pre-diabetes risk at the future or at follow up. In crude logistic regression analysis, MetS Z-score level at baseline positively predicted the risk of future diabetes (RR = 1.94, RR = 3.84) and pre-diabetes (RR = 1.5, RR = 2.17) in total population and female group, respectively (all $P < 0.05$). However, significant results were not detected in the male group. In multivariable logistic regression analyses, the adjustment was made for age and gender as confounding factors (model). As illustrated in Table 3, the associations remained significant for diabetes (RR = 2.69, RR = 4.01) and prediabetes (RR = 1.76, RR = 2.07) in total population and female group, respectively (all $P < 0.05$). However, such significant results were not observed in the male group.

ROC curve analysis was used to determine the cutoff value of MetS Z-score at baseline for predicting diabetes and prediabetes in at follow up. The area under the ROC curve of MetS Z-score for predicting the incidence of diabetes and prediabetes is shown in Figure 3. A cutoff value 0.97 for MetS Z-score was obtained for differentiating the female patients with diabetes from normal with corresponding specificity of 56% and sensitivity of 72% and area under the ROC curve (AUC = 0.67, 95%CI: 0.59-0.74; $P < 0.05$). A cutoff value 0.78 for MetS Z-score was obtained for differentiating the total population with diabetes from normal with corresponding specificity of 56% and sensitivity of 66% and area under the ROC curve (AUC = 0.63, 95%CI: 0.57-0.69; $P < 0.05$). A cutoff value of Met Z-score at 0.94 was obtained for differentiating the female patients with prediabetes from normal people, with a corresponding specificity 58% and sensitivity 60% and area under the ROC curve of (AUC = 0.6, 95%CI: 0.55-0.64; $P < 0.05$) Also, a cutoff value of MetS Z-score 0.52 was obtained for differentiating total patients with prediabetes from normal people, with a corresponding specificity 72% and sensitivity 42% and area under the ROC curve of (AUC = 0.58, 95%CI: 0.54-0.61; $P < 0.05$) (Table 4).

Table 1 Anthropometric, laboratory and clinical characteristics of first degree relatives of diabetes mellitus between normal and diabetic subjects in total population and sex difference

Variables	Total			Male			Female		
	Diabetes (*n* = 78)	Normal (*n* = 630)	*P* value	Diabetes (*n* = 26)	Normal (*n* = 160)	*P* value	Diabetes (*n* = 52)	Normal (*n* = 470)	*P* value
Age (yr)	43.33 ± 6.15	42.01 ± 6.14	0.075	45.15 ± 7.33	42.83 ± 6.65	0.1	42.42 ± 5.31	41.71 ± 5.93	0.4
BMI (kg/m^2)	29.33 ± 4.35	28.16 ± 4.20	0.02	27.6 ± 3.44	26.93 ± 3.64	0.38	30.16 ± 4.53	28.59 ± 4.31	0.01
Smoking, yes [*n* (%)]	2 (9.5%)	17 (8.03%)	0.84	1 (14.3%)	12 (25%)	0.53	1 (1.1%)	5 (2.3%)	0.43
Education (diploma and more)	33 (42.3%)	317 (51.5%)	0.12	13 (50%)	108 (68.8%)	0.06	20 (38.5%)	208 (45.5%)	0.33
WHR	0.83 ± 0.06	0.81 ± 0.07	0.01	0.9 ± 0.03	0.89 ± 0.05	0.62	0.8 ± 0.04	0.79 ± 0.05	0.04
Blood glucose 0 (mg/dL)	90.45 ± 6.92	87.05 ± 7.91	0.001	91.76 ± 5.58	87.79 ± 8.28	0.01	89.81 ± 7.45	86.8 ± 7.78	0.008
Blood glucose 30 (mg/dL)	143.92 ± 24.56	125.93 ± 24.91	0.001	144.32 ± 25.32	133.08 ± 27.98	0.06	143.73 ± 24.43	123.22 ± 23.14	0.001
Blood glucose 60 (mg/dL)	152.11 ± 34.83	121.58 ± 31.49	0.001	148.88 ± 38.67	129.25 ± 36.37	0.01	153.69 ± 33.06	118.74 ± 29.08	0.001
Blood glucose 120 (mg/dL)	108.93 ± 19.95	97.81 ± 21.05	0.001	102.93 ± 23.43	88.76 ± 22.44	0.003	111.88 ± 17.5	101.01 ± 19.6	0.001
HbA1c	5.2 ± 0.72	4.92 ± 0.78	0.006	5.28 ± 0.9	4.93 ± 0.63	0.03	5.16 ± 0.61	4.92 ± 0.82	0.05
Triglyceride (mg/dL)	181.42 ± 100.22	146.30 ± 81.10	0.001	203.48 ± 97.91	174.98 ± 96.79	0.17	170.6 ± 100.51	136.29 ± 72.42	0.002
Total cholesterol (mg/dL)	203.44 ± 44.55	189.96 ± 38.65	0.005	201.28 ± 40.22	192.09 ± 37.63	0.26	204.5 ± 46.87	189.37 ± 38.93	0.01
HDL (mg/dL)	44.21 ± 10.6	45.33 ± 11.57	0.43	43.34 ± 11.3	40.96 ± 10.82	0.32	44.6 ± 10.36	46.85 ± 11.46	0.18
Systolic pressure (cmHg)	11.58 ± 1.63	11.26 ± 1.48	0.08	12.14 ± 1.61	11.52 ± 1.49	0.06	11.32 ± 1.59	11.17 ± 1.47	0.48
Diastolic pressure (cmHg)	7.45 ± 1.24	7.41 ± 1.12	0.76	7.62 ± 1.16	7.55 ± 1.16	0.87	7.38 ± 1.28	7.36 ± 1.1	0.94

Values are presented as mean ± SD or *n* (%). BMI: Body mass index; WHR: Waist-to-hip ratio; HbA1c: Hemoglobin A1c; HDL: High-density lipoproteins.

DISCUSSION

To our knowledge, this is the first population-based study conducted to evaluate the association between MetS Z-score and the incidence of pre-diabetes/T2DM in the FDR population who were normal at first visit.

This study discovered that the degree of severity of MetS score as a linear measure is a predictive factor for the incidence of T2DM and prediabetes in the future. This association was overall moderate in the total population (AUC = 0.63) and mildly stronger in the females (AUC = 0.68) of the FDR population. Previously, MetS Z-score was correlated similarly to the prediction of CVD[13] and diabetes[12] in the non-FDR population. DeBoer *et al*[12] concluded that the severity of MetS in childhood could predict the incidence of adult T2DM in the future. In other studies, a strong correlation between MetS Z-score and HbA1c, fasting insulin and insulin resistance has been illustrated[28]. These findings suggest that MetS Z-score can potentially be used to detect risk and follow T2DM progression over time[12].

It has been reported that childhood MetS Z-score can predict diabetes risk in the future with an OR of 2.7 by the mean age of 38.5[12]. We found similar results: In the total FDR population, the RR for each 1.0 unit increase in the adulthood MetS Z-score in predicting diabetes and pre-diabetes by a mean age of 43 was 2.69 and 1.76 respectively.

Besides, MetS Z-score is associated with childhood obesity and is a significant risk

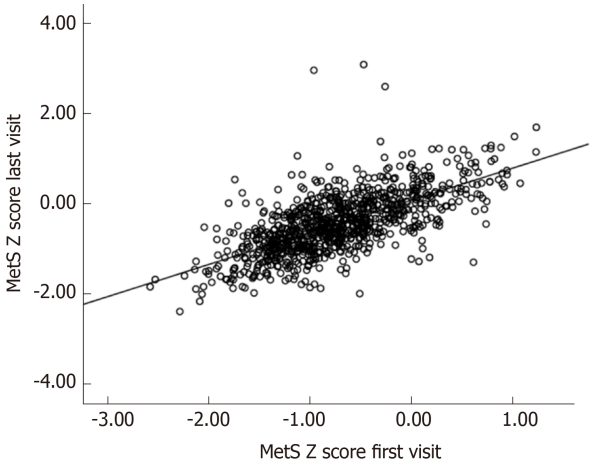

Figure 1 Correlation of metabolic syndrome severity scores within individuals over time. Metabolic syndrome severity Z-scores on the x-axis in first visit (2003-2005) and on the y-axis during last visit (2018). Pearson's $r = 0.67$ ($P < 0.001$).

factor for prediabetes and progression to T2DM[29]. Currently, there is an epidemic of obesity in the world which begins early in life. Tools such as MetS Z-score can be utilized to diagnose the population at higher risk for future disease and assisting primary prevention by suggesting lifestyle modifications[5].

Measuring MetS has its limitations. Firstly, it is difficult to monitor changes in MetS over time[30,31]. Secondly, despite evidence demonstrating that elevated WC or high triacylglycerol levels have a more important role and stronger association with MetS risk over time due to abnormal cellular processes involved, in this method of measurement equal importance is given to all of the components of MetS[32,33]. Thirdly, gender, race and ethnicity cause variation in the MetS value, for example, African American men have a low prevalence of MetS despite having high rates of T2DM and death from cardiovascular disease[34-36].

All of the available data establishes the necessity of using a continuous measurement of MetS for clinical applications. Gurka *et al*[37,38] have formulated sex- and race/ethnicity-specific MetS Z-score. The standardized Z-scores for each component coming together creates an overall estimate of the severity of MetS[24] with a linear association with future risk of T2DM and offers a tool for monitoring treatment efficacy[12].

The role of genetic factors in MetS cannot be ignored[15]. The association of oxidative stress with inflammatory processes and MetS Z-score has been studied[5,39]. In our study, the high correlation between these scores over 15 years suggests a degree of consistency of MetS in a given individual over time or a genetic susceptibility in the FDR population.

Several studies on FDR individuals indicated that alteration in carbohydrate and lipid metabolism including central obesity, dyslipidemia, glucose intolerance, and high blood pressure start at an early age[40,41]. Siewert *et al*[19] illustrated that the prevalence of MetS is high in young FDR adults, and since MetS and T2DM are closely related diseases and are driven by the same metabolic disturbances, preventive measures at an early age seem appropriate.

The prevalence of MetS is influenced by various factors such as gender, environmental and cultural in addition to genetic factors[42].

Similar to the USA the prevalence of MetS is higher in women than men in Iran[43,44]. This sex difference can be explained by a statistically significant higher prevalence of MetS components in women and agrees with the results of our study. In the FDR population, women with higher WHR, TG, and cholesterol than normal women had a higher chance to progress to diabetes or prediabetes while such a difference was not observed in male FDRs. The differences in lipid profile could be explained by hepatic lipase activity, patterns of diet and physical activity. Women show a sharp decline in physical activity at adolescence as compared to men, and this could explain the higher prevalence of obesity in women[43].

Recently, during one cohort study in Iran, the paternal history of T2DM was independently associated with increased risk for pre-diabetes/T2D in adolescence [HR = 1.63 (1.02–2.60)][45]. One of our other studies reported that the glycemic response to OGTT may predict the risk of development to T2DM in the FDR population[46].

Table 2 Anthropometric, laboratory and clinical characteristics of first degree relatives of type 2 diabetes mellitus between normal and pre-diabetes subjects at total population and sex categories

Variables	Total			Male			Female		
	Pre-diabetes (*n* = 344)	Normal (*n* = 630)	*P* value	Pre-diabetes (*n* = 94)	Normal (*n* = 161)	*P* value	Pre-diabetes (*n* = 250)	Normal (*n* = 470)	*P* value
Age (yr)	43.04 ± 6.33	42.01 ± 6.14	0.01	43.2 ± 6.12	42.83 ± 6.65	0.66	42.98 ± 6.42	41.71 ± 5.93	0.008
BMI (kg/m^2)	28.44 ± 4.06	28.16 ± 4.2	0.32	26.84 ± 3.56	26.93 ± 3.64	0.84	29.04 ± 4.09	28.59 ± 4.31	0.17
Smoking, yes [*n* (%)]	8 (7.8%)	17 (8.3%)	0.88	7 (21.2%)	12 (25%)	0.69	1 (1.4%)	5 (3.2%)	0.45
Education (diploma and more)	154 (45.4%)	317 (51.5%)	0.07	61 (66.3%)	108 (68.8%)	0.68	93 (37.7%)	208 (45.5%)	0.05
WHR	0.82 ± 0.06	0.81 ± 0.07	0.04	0.9 ± 0.05	0.89 ± 0.05	0.83	0.8 ± 0.05	0.79 ± 0.05	0.01
Blood glucose 0 (mg/dL)	89.69 ± 6.9	87.05 ± 7.91	0.001	90.57 ± 6.7	87.79 ± 8.28	0.06	89.37 ± 6.96	86.8 ± 7.78	0.001
Blood glucose 30 (mg/dL)	136.63 ± 26	125.93 ± 24.91	0.001	141.44 ± 30.23	133.08 ± 27.98	0.02	134.74 ± 23.94	123.22 ± 23.14	0.001
Blood glucose 60 (mg/dL)	135.68 ± 31.23	121.58 ± 31.49	0.001	134.98 ± 33.37	129.25 ± 36.37	0.21	135.94 ± 30.45	118.74 ± 29.08	0.001
Blood glucose 120 (mg/dL)	104.2 ± 21.36	97.81 ± 21.05	0.001	94.72 ± 23.52	88.76 ± 22.44	0.04	107.7 ± 19.41	101.01 ± 19.6	0.001
HbA1c	5.06 ± 0.7	4.92 ± 0.78	0.009	5.09 ± 0.83	4.93 ± 0.63	0.1	5.05 ± 0.65	4.92 ± 0.82	0.04
Triglyceride (mg/dL)	157.78 ± 81.03	146.3 ± 81.1	0.03	176.98 ± 90.75	174.98 ± 96.79	0.87	150.56 ± 76.02	136.29 ± 72.42	0.01
Total cholesterol (mg/dL)	193.34 ± 38.2	189.96 ± 38.65	0.19	188.09 ± 36.58	192.09 ± 37.63	0.41	195.3 ± 38.67	189.37 ± 38.93	0.05
HDL (mg/dL)	44.37 ± 45.33	10.98 ± 11.57	0.21	41.26 ± 11.46	40.96 ± 10.82	0.83	45.51 ± 10.6	46.85 ± 11.46	0.13
Systolic pressure (cmHg)	11.67 ± 1.65	11.26 ± 1.48	0.001	11.75 ± 1.73	11.52 ± 1.49	0.26	11.64 ± 1.63	11.17 ± 1.47	0.001
Diastolic pressure (cmHg)	7.6 ± 1.14	7.41 ± 1.12	0.01	7.75 ± 1.19	7.55 ± 1.16	0.19	7.55 ± 1.12	7.36 ± 1.1	0.03

Values are presented as mean ± SD or *n* (%), *P* values resulted from independent *t*-test for quantitative and χ^2 for categorical data. BMI: Body mass index; WHR: Waist-to-hip ratio; HbA1c: Hemoglobin A1c; HDL: High-density lipoproteins.

In summary, our study has suggested that MetS Z-score at baseline, in FDR of T2DM patients with normal glucose tolerance, predicts the incidence of future diabetes and prediabetes. In this study, the cutoff values of MetS Z-score for predicting prediabetes and diabetes were 0.94 and 0.97, respectively. This negligible difference between two groups in terms of cutoff values highlights the importance of intervention at the prediabetes stage. Elevated levels may also be used to motivate patients to increase their physical activity or adopt a healthy diet to reverse the prediabetic state[47]. Appropriate interventions at an earlier stage in MetS may be considered as an effective strategy for preventing the development of diabetes and prediabetes in such a high-risk population.

Table 3 Relationship between metabolic syndrome severity Z-score and risk of diabetes and pre-diabetes in first degree relatives of type 2 diabetes mellitus

	Diabetes, RR (95%CI)			Pre-diabetes, RR (95%CI)		
	Total	Male	Female	Total	Male	Female
Crude model	1.94 (1.33-2.82)[a]	1.41 (0.65-3.03)	3.83 (2.01-7.29)[a]	1.5 (1.2-1.87)[a]	1.28 (0.82-1.99)	2.17 (1.53-3.08)[a]
Model 1[1]	2.69 (1.61-4.48)[a]	1.04 (0.69-3.4)	4.01 (2.07-7.76)[a]	1.76 (1.33-2.33)[a]	1.31 (0.83-2.05)	2.07 (1.45-2.97)[a]

[1]Age–Sex adjusted for total population and only age adjusted for male and female groups.
[a]$P < 0.05$. RR: Relative risk; CI: Confidence interval.

Table 4 Area under the curve, sensitivity and specificity of metabolic syndrome severity Z-score for predicting the risk of affecting by diabetes and pre-diabetes in future for first degree relatives of type 2 diabetes mellitus when they are normal glucose tolerance at the begging of study

	Diabetes			Pre-diabetes		
	Total	Male	Female	Total	Male	Female
AUC (95%CI)	0.63 (0.57-0.69)[a]	0.55 (0.43-0.68)	0.67 (0.59-0.74)[a]	0.58 (0.54-0.61)[a]	0.55 (0.47-0.62)	0.6 (0.55-0.64)[a]
Sensitivity (%)	66	76	72	42	71	60
Specificity (%)	56	43	56	72	44	58

[a]$P < 0.05$. AUC: Area under the curve.

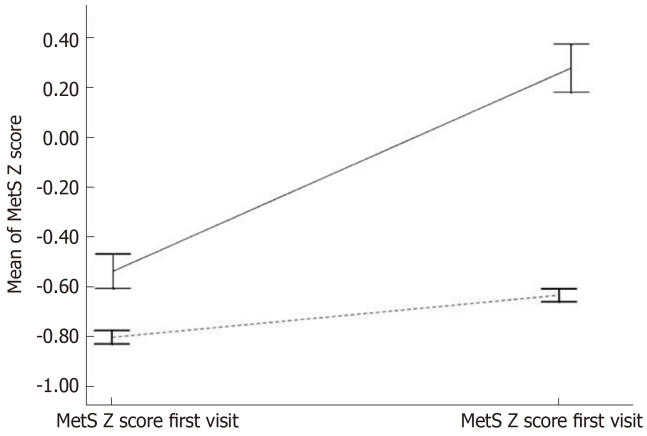

Figure 2 Mean metabolic syndrome severity scores within individuals by later diabetes status. Metabolic syndrome Z-score (mean, 95% confidence interval) by disease status for diabetes. Scores shown are those obtained during first and last visits among individuals who remained disease-free (dotted line, $n = 564$), those with incident disease between two visits (continuous line, $n = 70$). Comparison with disease-free group: $P < 0.05$ for first visit, $P < 0.01$ for last visit.

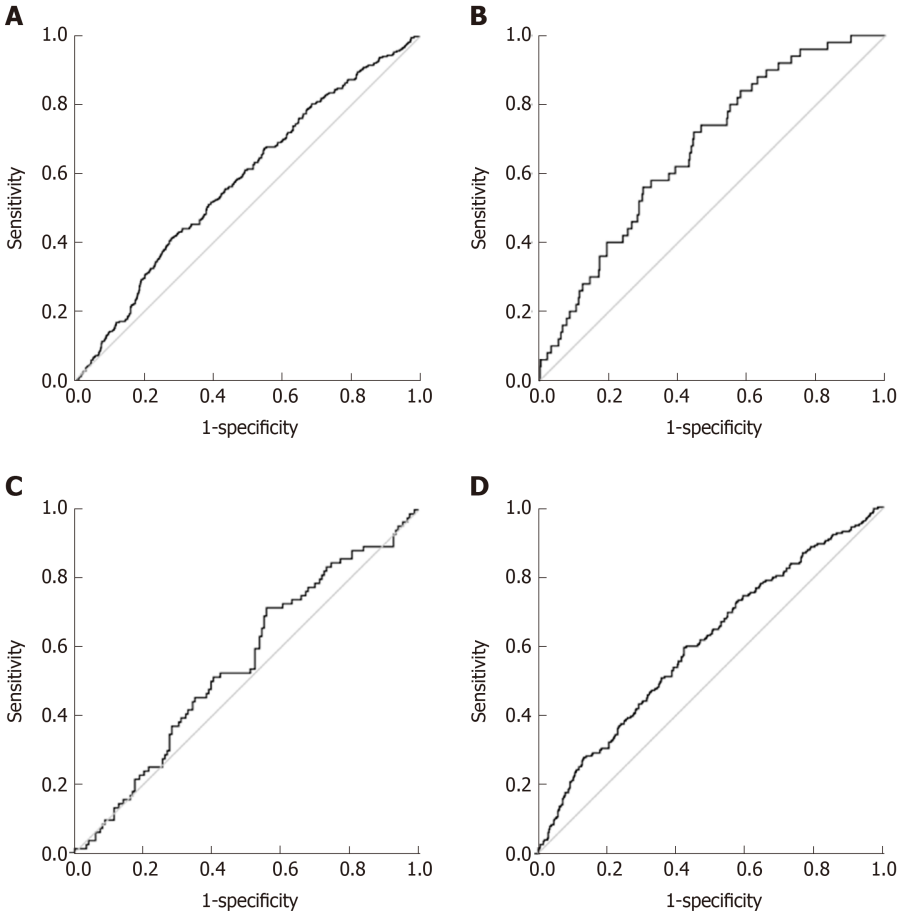

Figure 3 Receiver operating characteristic curves of metabolic syndrome Z-score. A and B: Predicting future incidence of diabetes (A: Male; B: Female); C and D: Predicting future incidence of prediabetes (C: Male; D: Female).

ARTICLE HIGHLIGHTS

Research background

There is potential links between MetS Z-score as a marker for prediabetes or type 2 diabetes mellitus (T2DM) risk.

Research motivation

Iran is a developing country and its population is adopting a more sedentary lifestyle with new diets resulting in a high prevalence of T2DM and prediabetes.

Research objectives

In this study, the association the of severity of MetS Z-score in FDRs of T2DM was assessed with the risk of prediabetes and type 2 diabetes in future.

Research methods

In a prospective cohort study during a long-term follow-up period for the first time in Iran and as one of scare studies around the world we evaluated the predictive role of MetS Z-score for prediabetes and diabetes incidence risk in future among normal glucose tests. Our study results help clinicians to identify and treat this high-risk population through conducting of interventions for preventing MetS or diminishing its side effects.

Research results

MetS Z-score at the baseline, is a significant predictor for developing future T2DM and prediabetes in total population and female group. Reliable cut off values with high accuracy were obtained in the receiver operating characteristic curve analysis based on the MetS Z-score for differentiating patients with diabetes and prediabetes from the normal population.

Research conclusions

MetS Z-score is a significant predictor for incidence of diabetes and prediabetes risk in future in high risk population of FDR and cut off value for MetS score was not notably different for those people who affected by diabetes and prediabetes. This negligible difference between two groups in terms of cut off values highlights the importance of intervention at the prediabetes stage.

Research perspectives

The FDR people with high risk of developing diabetes and prediabetes are identifiable based on MetS Z-score. Accordingly, appropriate interventions at an earlier stage in MetS may be considered as an effective strategy for preventing the development of diabetes and prediabetes in such a high-risk population.

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