DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Mechanisms underlying peripheral resistance in a rat model of prediabetes

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Eugénia Carvalho (Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e do Professor Doutor Rui Carvalho (Universidade de Coimbra).

Manuela Gachineiro Cerqueira

2015

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Mechanisms underlying peripheral resistance in a rat model of prediabetes

2015

TABLE OF CONTENTS

ACKNOWLEDGMENTS

À FCT - Fundação para a Ciência e a Tecnologia, comparticipada pelo Fundo FEDER através do Programa Operacional Factores de Competitividade – COMPETE e por fundos nacionais do Ministério da Educação e Ciência, no âmbito dos projecto UID/NEU/04539/2013 e EXCL/DTP-PIC/0069/2012. Agradeço também à SPD/GIFT.

À Dra. Eugénia Carvalho, agradeço a oportunidade dada ao acolher-me no seu grupo de trabalho, a exigência que me ensinou a querer ser melhor e a motivação e confiança que me depositou desde o início. Obrigada por promover a minha a vontade de continuar!

Ao Dr. Flávio Reis, pela disponibilidade, pelos ensinamentos, pelas sugestões e pelas palavras de incentivo.

Ao Dr. Rui Carvalho, por ter aceite ser meu orientador, pela disponibilidade e pelas palavras de incentivo. Obrigada por ser um excelente professor e querer sempre mais para os seus alunos.

À Ana Burgeiro, pela disponibilidade, paciência para as dúvidas, pela altruísta vontade de me ensinar e pelas palavras de apoio. Foi um prazer trabalhar contigo.

À Sara Nunes, por todo o apoio, principalmente no inicio deste trabalho, pelos ensinamentos e incentivo. Desejo-te a maior sorte do mundo!

Ao Fábio Carvalho pela paciência que teve para me ensinar tudo o que sabia no meu início e por estar sempre disponível a responder a qualquer dúvida.

Ao Ermelindo, à Tatiana e ao Abdullah, pela receptividade, convivência no laboratório e pela prontidão em ajudar.

À Cristina Carvalho, pela simpatia e disponibilidade em responder a qualquer dúvida.

A todos os professores com quem me cruzei, que, com a partilha da sua experiência me fizeram querer este caminho e a conseguir aqui chegar.

Ao Oreo, pelo companheirismo, por todas as vivências e memórias que ficarão para a vida, pela importância que tens, por mais longe que estejas.

À Susana Comprido, por lá estares, sempre. Pela paciência, ajuda e amizade. Que seja assim, sempre.

À Mel, à Letícia, ao Jugo, ao Mina, ao Amarelo, ao Joca, ao Ivo, ao Thony, ao Mykola, ao Nascimento, ao Rojões, ao Mémé, à Sara e ao Capela, por terem feito parte desta caminhada, pela amizade e pelos bons momentos que passamos nestes últimos anos.

Ao Emanuel, por te teres tornado um grande amigo.

Ao Gui, pela presença nos bons e maus momentos. Pela amizade, pelo companheirismo e amor. Obrigada por estares em todas as memórias.

Aos pais do Gui, António Loureiro e Dina Loureiro, por me acolherem e por estarem sempre prontos a ajudar.

A Coimbra, pelas vivências, pelas boas memórias e pelas pessoas com quem nela me cruzei.

Ao Zé Manel e ao Benoit por fazerem parte da família e pelos momentos alegres.

Às minhas irmãs Susana e Martinha e ao meu irmão Leonel, por serem mais do que irmãos, pelo carinho e pelo apoio incondicional.

Aos meus sobrinhos, Tiago, Mariana, Matias e Joaquim, pelas brincadeiras, pelas gargalhadas e por serem sempre o melhor do mundo. Que a vida vos dê o melhor.

Ao papá e à mamã, pelo amor, pelo trabalho e pela educação. Obrigada por toda a confiança e investimento depositados em mim e por terem sempre ajudado a alcançar os meus objectivos. Sem vocês nada disto seria possível.

ABSTRACT

Type 2 diabetes mellitus (T2DM), a chronic metabolic disease, is reaching epidemic proportions and is becoming a worldwide health problem. Despite 30-50% of the diabetic population remaining undiagnosed, nowadays, almost 400 million people suffer from this disease with consequent severe complications. Major causes are lifestyle and diet habits, practicing less exercise and westernizing eating habits, including increased consumption of sugars. Retinopathy, nephropathy, neuropathy and cardiovascular diseases resulting from insulin resistance, hyperglycemia, dyslipidemia, hypertension, systemic inflammation and oxidative stress, are common micro-and macrovascular complications observed in T2DM patients. These metabolic alterations can start developing years before the onset of diabetes; therefore, already in the prediabetic state, characterized by a slight increase in fasting plasma glucose levels, it is possible to observe many abnormalities associated with T2DM.

In order to understand the molecular mechanisms underlying insulin resistance development in the prediabetic state, we used a prediabetic animal model consisting of a sucrose enriched diet (HSu) (35%) during nine weeks. The potential impairment in glucose and lipid metabolism evoked by the HSu diet was evaluated in isolated adipocytes, liver and skeletal muscle.

Our results revealed a significantly altered glucose excursion during a glucose tolerance test (GTT) in the HSu treated rats. In addition, the insulin-stimulated glucose uptake in isolated adipocytes was significantly reduced in the same animals, as compared to controls. Moreover, several important nodes of the insulin signaling cascade were also modulated by the chronic treatment with HSu

diet, including hepatic glucose transporter 1, glucose-6-phosphatase and fatty acids synthase.

In conclusion, our findings indicate that a HSu diet might induce at least in part impaired glucose tolerance and decreased insulin-stimulated glucose uptake in fat cells, together with impaired gluconeogenesis and adipogenesis. These results support the idea that the body begins to resent unhealthy lifestyles long before the onset of the disease and that prediabetes might be viewed as the main target state to prevent the development of T2DM.

Keywords: prediabetes; insulin resistance; high-sucrose diet.

RESUMO

A diabetes mellitus do tipo 2 (DMT2) é uma doença metabólica grave, cuja incidência tem vindo a aumentar a cada ano, alcançando proporções epidémicas e tornando-se um problema de saúde a nível global. Esta tendência Mundial deve-se essencialmente ao facto de a população estar a mudar o seu estilo de vida, incluindo a praticar menos exercício físico, a ocidentalizar a sua alimentação, regendo-se por uma dieta mais rica em hidratos de carbono simples. Apesar de 30 a 50% das pessoas que sofrem de DMT2 permanecerem ainda por diagnosticar, atualmente cerca de 400 milhões de pessoas sofrem desta doença e das complicações a ela associadas. As principais complicações micro e macrovasculares são a retinopatia, a nefropatia e a neuropatia diabéticas, e as doenças cardiovasculares, que resultam de fenómenos como a resistência à insulina, a hiperglicémia, a dislipidemia, a hipertensão arterial, a inflamação sistémica e o stress oxidativo. Essas alterações metabólicas começam a desenvolver-se anos antes do início da diabetes; com efeito, num estado de pré-diabetes, caraterizado por um aumento subtil da glicemia, já é possível observar alterações características da DMT2.

Com o objectivo de compreender os mecanismos moleculares subjacentes ao fenómeno de resistência à insulina num estado de pré-diabetes, estudámos um modelo animal obtido através de uma dieta enriquecida em sacarose (35%) durante 9 semanas. Potenciais alterações, advindas desta dieta, no metabolismo da glucose e dos lípidos foram avaliadas através de estudos em tecido adiposo epididimal, fígado e músculo esquelético.

Os nossos resultados mostraram uma alteração significativa no teste de tolerância à glicose nos ratos pré-diabéticos. Paralelamente, verificou-se uma redução significativa da captação de glicose em adipócitos isolados nos animais tratados, comparativamente aos controlo. A cascata de sinalização da insulina no grupo pré-diabético também revelou algumas alterações, nomeadamente ao nível do transportador de glucose 1, da Glucose-6-fosfatase e da enzima que intervém na síntese de ácidos gordos (FAS).

Em conclusão, os nossos achados indicam que uma dieta enriquecida em sacarose pode induzir intolerância à glucose e redução da sua captação mediada pela insulina em adipócitos, bem como perturbações na gluconeogénese e na adipogénese. Estes resultados fortalecem a ideia de que o organismo começa a ressentir as alterações do estilo de vida muito antes do início da diabetes e que a pré-diabetes deve ser encarada como a etapa crucial de intervenção para prevenir o desenvolvimento de DMT2.

Palavras-chave: pré-diabetes; resistência à insulina; dieta rica em sacarose.

LIST OF ABREVIATIONS

ACC1	Acetyl-CoA carboxylase 1
ADA	American Diabetes Association
AS160	Rab-GTPase-activating protein
AUC	Area under the curve
BCA	Bicinchoninic acid
BMI	Body mass index
BW	Body weight
C	Control
ChREBP	Carbohydrate response element binding protein
CVD	Cardiovascular disease
DCCT	Diabetes Control and Complication Trial
DGAT1	Diacylglycerol acyltrasferase 1
DM	Diabetes mellitus
DMT2	*Diabetes mellitus do tipo 2*
ECF	Enhanced chemifluorescence
EDTA	Ethylenediamine tetraacid
EGP	Endogenous glucose production
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein-1
ER	Endoplasmatic reticulum
F-1,6-Pase	Fructose-(1,6)-biphosphatase
F6P	Fructose-6-phosphate
FAS	Fatty acid synthase

FBPase	Fructose 1,6 bisphosphatase
FDR	First degree relatives
FFA	Free-fatty acids
FoxO	Forkhead box O1
G-6-Pase	Glucose-6-phosphatase
GDM	Gestational diabetes mellitus
GK	Glucokinase
GLUT	Glucose transporter
GS	Glycogen synthase
GSK3	Glycogen synthase kinase-3
GTT	Glucose tolerance test
HbA1c	Glycated hemoglobin
HDL	High density lipoprotein
HSL	Hormone-sensitive lipase
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IR	Insulin receptor
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
MODY	Maturity-onset diabetes of the young
mTOR	Mammalian target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NGSP	National Glycohemoglobin Standardization Program
NEFAs	Non-esterified free fatty acids

OGTT	Oral glucose tolerance test
P70 S6K	P70 ribossomal protein S6 kinase
PCOS	Polycystic ovarian syndrome
PDK1	Phosphoinositide-dependent protein kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PGC1	Proliferator-activated receptor gamma coactivator1
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol-3, 4, 5-triphosphate
PKB	Protein kinase-B
PMSF	Phenylmethylsulfonyl fluoride
PPAR α	Peroxisome proliferator-activated receptor alpha
PPARβ	Peroxisome proliferator-activated receptor beta
PPARγ	Peroxisome proliferator-activated receptor gamma
PTP1B	Protein-tyrosine phosphatase 1B
PVDF	Polyvinylidene fluoride
Rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SREBP-1	Sterol regulatory element-binding protein-1
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGs	Triglycerides
TSC	Tuberous sclerosis complex
VLDL	Very low density lipoprotein

WB	Western blot

I. Introduction

I. INTRODUCTION

1. Diabetes

1.1 Epidemiology

Diabetes is one of the most common non-communicable diseases, in developed countries [1]-[2], directly correlated with economic and industrial development [3].

Elevated socio-economic costs are one of the worldwide consequences of diabetes mellitus (DM) due to premature morbidity and mortality of those who have the disease. The intrinsic metabolic alterations of DM begin much earlier than the manifestation of the disease itself, so many years of deterioration of the organism pass before the diagnosis [4]. Also, DM is a disease that decreases the quality of life of patient, also decreasing their life expectancy by at least ten years [5]. In 2014, diabetes caused 4.9 million deaths, every 7 seconds a person died from this disease [6].

In 2014, 387 million individuals had DM, and even more worrying is the fact that 30 to 50% of the diabetic population remains undiagnosed making these numbers an underestimation [5] - [6].

In addition, the last report of the International Diabetes Federation (IDF) indicates an expected increase in the number of diabetics by an additional 205 million until 2035, raising the current number of 387 million to 592 million (Figure 1). This increase will be more pronounced in Africa and the

Western Pacific due to their late lifestyle modification toward a Western diet also in addition to lack of exercise [6], [7].

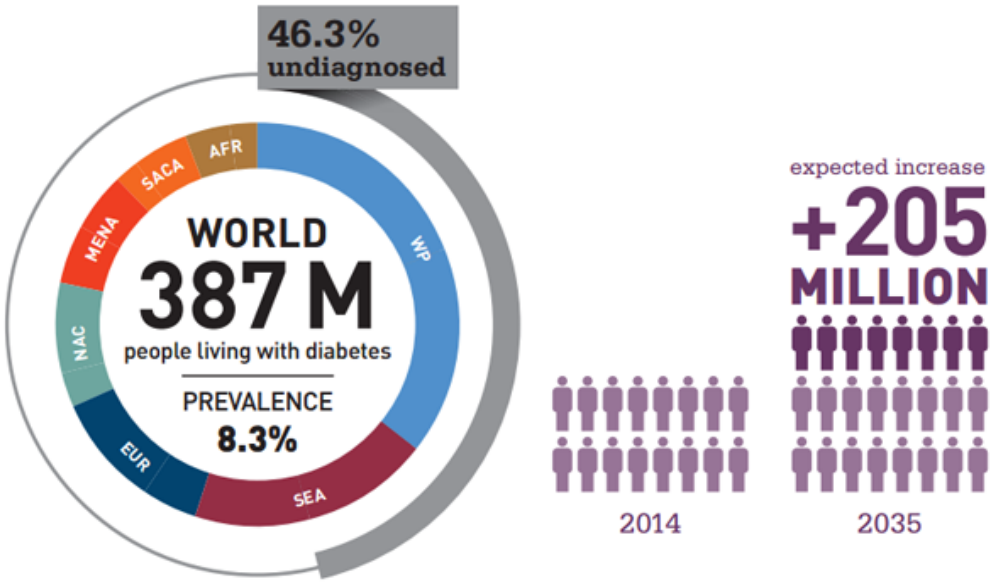

Figure 1. Wordlwide diabetes incidence. (http://www.idf.org)

In Portugal, in 2013, 40% of the population between the ages of 20 and 79 were diagnosed as prediabetic or diabetic. Specifically, 13% of individuals had diabetes and 27% already had prediabetes (Figure 2) [8]. In addition, both prediabetes and diabetes have been increasing in prevalence since 2009, when the percentages were 11.7% and 23.2%, respectively [9].

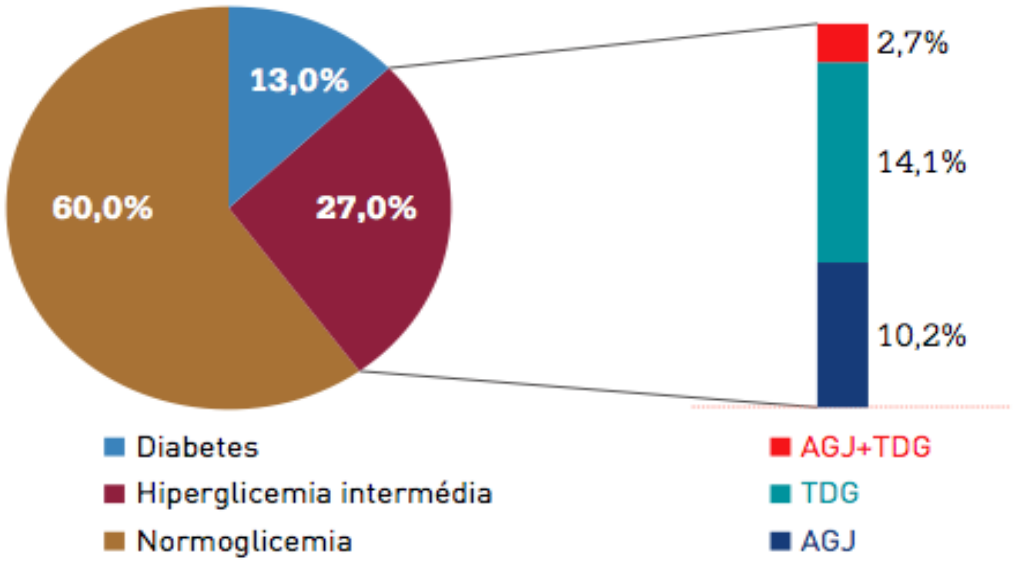

Figure 2. Prediabetes and diabetes prevalence in Portugal in 2013. (TDG – Impaired glucose tolerance; AGJ – Impaired fasting Glucose). (Observatório Nacional da Diabetes – Relatório Anual do Observatório Nacional da Diabetes 2013).

1.2 Classification

Diabetes can be classified in four general categories [4]:

a) Type 1 diabetes mellitus (T1DM)

Due to beta-cell destruction, usually leading to absolute insulin deficiency.

b) Type 2 diabetes mellitus (T2DM)

Due to a progressive insulin secretory defects, as well as insulin resistance.

c) Gestational diabetes mellitus (GDM)

Diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes.

d) Specific types of diabetes due to other causes

Neonatal diabetes and maturity-onset diabetes of the young [MODY]), diseases of the exocrine pancreas (such as cystic fibrosis), and drug or chemical-induced diabetes, such as in the treatments used for HIV/AIDS or immunosuppression after organ transplantation.

1.2.1 Type 1 diabetes mellitus

According to the American Diabetes Association (ADA), this form of diabetes accounts for a small percentage, 5-10%, of the total diabetes cases [1]. T1DM results from an autoimmune reaction to proteins of the pancreatic beta-cells, and their consequent destruction. Moreover, insulin resistance can also be present [4]. Insulin therapy is needed in order to keep the survival of patients [10].

T1DM appears usually early in life, between 4-5 years of age. It can also be diagnose during the teenage years or even appear in some cases in early adulthood [7] and the most common symptoms are:

- Abnormal thirst and a dry mouth
- Frequently urination
- Lack of energy, extreme tiredness
- Constant hunger
- Sudden weight loss
- Slow-healing wounds
- Recurrent infections
- Blurred vision

However, T1DM patients can normally have a common healthy life with a combination of daily insulin therapy, close monitoring of glucose levels, healthy diet and regular physical exercise [11].

1.2.2 Type 2 diabetes mellitus

T2DM is the most prevalent type form of diabetes, affecting 90-95% of all the diabetic population [1]. It has also been known as non-insulin-dependent diabetes. It is characterized by insulin resistance and in some cases, deficient insulin secretion. Usually T2DM patients do not need insulin treatment to control the disease [4]. The etiology of T2DM is not clearly known, however, there are some inherent characteristics, such as lifestyle choices, physical inactivity and obesity (Figure 3) but the symptoms are well known (Figure 4).

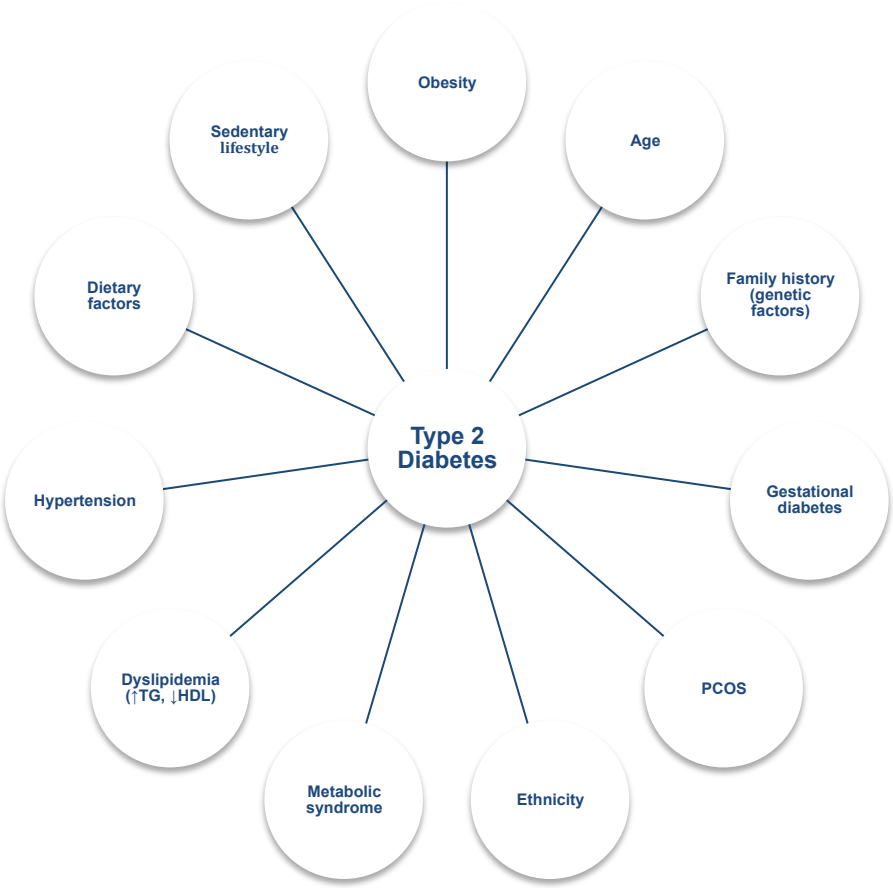

Figure 3. Risk factors in the development of T2DM. HDL, high-density lipoprotein cholesterol; PCOS, polycystic ovarian syndrome; TG, triglycerides [10]**.**

Figure 4. T2DM symptoms. (http://www.stopchildhoodobesity.com)

Furthermore it is possible to see a correlation between the epidemic rise in obesity during the last decade and the increase in individuals with

T2DM. Obesity, particularly visceral obesity, together with physical inactivity, can lead to insulin resistance and ultimately to the diabetic state particularly in those with deficiency in insulin secretion [10]. Advanced age is also one of the factors that can predispose to diabetes, probably because it is correlated with greater levels of physical inactivity. First-degree relatives (FDR) are more susceptible to developing diabetes at an earlier stage, because of the shared features of lifestyle, and in a more severe form, resulting from the genetics involved and from the western lifestyle characteristics, such as poor diet and lack of exercise [12]. Also, studies [13], have shown a decrease (>50%) in the insulin receptor substrate (IRS1) of FDR individuals when compared to a matched control group and, this decrease in the IRS1 is also associated to a markedly insulin resistance.

FDR of diabetic patients also have an increased risk of developing diabetic complications. The risk can reach 50% [10] since they have a greater chance to carry the genetic predisposition for the disease [13] - [14] and its complications.

Non-diabetic FDR have been extensively studied, once they present some of the metabolic alterations found in their diabetic relatives, like insulin resistance, beta-cell dysfunction, obesity and impaired glucose tolerance, compared with healthy subjects without a family history of diabetes [16], [17]. Those FDR subjects are also important to search others alterations that can be used as markers for T2DM and predict the development of this disease [18].

It seems that insulin resistance may be the first alteration present in the disease, and this alteration could be already observed in lean offspring of

T2DM patients at high risk for the development of this disease, with ages rounding twenty three years of age, with similar anthropometric measurements, among, body mass index (BMI), waist-to-hip ratio and percent body fat, and with normal glucose tolerance [19]. Also, reduced insulin sensivity is related to early alterations in adipogenesis of non-obese first-degree relatives of subjects with T2DM, who has presented enlarged adipose cells, relating this alteration to insulin resistance [20].

1.2.3 Gestational diabetes mellitus

GDM generally appears in the latter half of gestation. Obesity, advanced maternal age, family history of T2DM, previous history of GDM or complications in a previous pregnancy are the risk factors for this type of diabetes which is characterized by a carbohydrate intolerance that can be of different severity [21]. This diabetic manifestation could bring several complications to the mother and to the fetus in the short and long term. The fetus can suffer from hyperbilirubinaemia, and hypoglycaemia, which can lead to serious neurologic injuries [22]. Moreover, hyperglycaemia could also surge as a result from the maternal hyperglycaemia causing increased fetal body mass causing difficulties during delivery and deterioration of pulmonary maturation, and therefore, respiratory distress syndrome. In addition, the fetus can develop obesity and diabetes just as the mother who also has an increased risk of around 50%, for T2DM. Hyperglicemia is present in 13% of pregnant women, per year, 0.1% have T1DM, 2-3% have T2DM and 12% have GDM, making this the most common problem during pregnancy [23]. It

has been described that 50% of the women who had T2DM during pregnancy already had GDM during a previous pregnancy. Therefore, it is extremely important to do an early diagnosis of GDM in order to prevent the development of other severe forms of diabetes, by introducing lifestyle changes to both the mother and the child [24].

1.2.4 Specific types of diabetes due to other causes

Monogenic diabetes is the outcome of one or more mutations that occurs on a single gene, mutations can be dominantly or recessively inherited. This form of diabetes only accounts for 1-2% of total diabetic cases and 90% of the time are misdiagnosed as T1DM or T2DM. They're due to monogenic defects that cause beta-cell dysfunction and are characterized by hyperglycaemia at an early age [10].

Neonatal diabetes is diagnosed during the first 6 months of life, resulting of mutations of genes involved in beta-cell development and function and it could be permanent or transient, taking into account its cause. This is rare form of diabetes, affecting 1 in 100 000-200 00 live births [10]. A permanent forms is commonly due to a defect in the gene encoding the Kir6.2 subunit of the beta-cell K_{ATP} channel while the more transient form of this disease is a defect on ZAC/HYAMI imprinting [4]. The transient form is a result of abnormalities in chromosome 6 and is characterized by low birth weight and umbilical hernia. Those patients are treated with insulin for about twelve weeks until they're treated, however, later in life, 50-60% of the cases diabetes returns resulting of beta-cell dysfunction [10].

Maturity-onset diabetes of the young (MODY) is an autosomal dominant form of inherited diabetes not dependent of insulin, normally diagnosed before the age of twenty-five and it can be now related with mutations in, at least, eight genes. It has also been characterized by impaired insulin secretion but slight or no defects in insulin action. Those mutated genes intervene in the encoding of glucose sensing enzyme glucokinase (GK) and in several transcription factors that affects beta-cell development and function. Glucokinase is an important enzyme involved in glucose metabolism in beta cells and in the liver, converting glucose to glucose-6-phosphate, which will stimulate insulin secretion by beta-cells [4], [7], [10].

2. Diabetes and complications

DM is one of the most concerning health problems worldwide. Diabetes is a metabolic disorder that has multiple causes, such as genetics and environmental factors, like obesity and sedentary lifestyle. It is characterized by chronic hyperglicemia and alterations in carbohydrate, fat and protein metabolism, due to an impairment on insulin action, insulin secretion or even both [10].

These metabolic changes, can in the long term, result in other serious complication: the microvascular, such as diabetic retinopathy, diabetic nephropathy and diabetic neuropathy that can dangerously progress to foot ulcers and amputation; and the macrovascular, like cardiovascular disease due to insulin resistance, hyperglicemia, dyslipidemia, hypertension, systemic inflammation and oxidative stress (Figure 5.) [25].

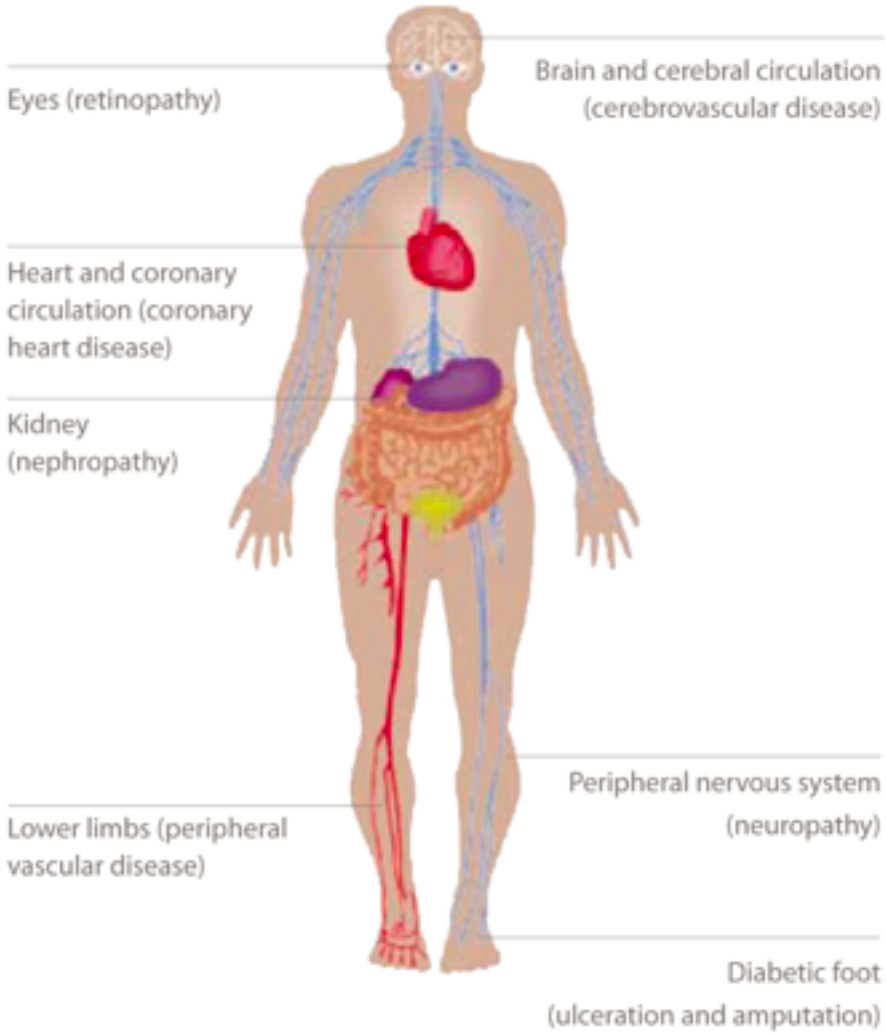

Figure 5. Major diabetic complications. (http://www.leicestershirediabetes.org.uk)

3. Diabetes-associated healthcare costs

The economic burden of DM for the healthcare system should be an incentive to change, betting on prevention instead treatment.

According to ADA, in USA is estimated that the healthcare costs of diabetes increased from 174 billions to 245 billions in 2012 (US Dollars) [26], numbers that not only reflect the increasing epidemic as the urgency in stop it.

In Portugal, in 2013, and according to the numbers of the 6th edition of the IDF Diabetes Atlas, healthcare spent on diabetes was around 1,713 million euros, 10% of the total healthcare expenses [9].

4. Insulin resistance

Insulin resistance is a major risk factor for the development of T2DM and it is caused by a reduced response of insulin-target tissues to the stimulation of this hormone [27]–[29]. Both in the fasting and in the fed state, peripheral glucose uptake, suppression of serum triglyceride production by very low density lipoprotein (VLDL), usually mediated by insulin are impaired [10], [13], [28]. Skeletal muscle is the first tissue that presents defects in insulin action, involving the glycogen synthetic pathway, which has been showed by studies in the offspring of two diabetic parents, revealing that insulin resistance in these individuals is of similar degree to that seen in type 2 diabetic patients [27], [28].

In adipose tissue, insulin resistance increases lipolysis which will increase the release of non-esterified free fatty acids (NEFAs), that, in turn, will act on the liver and skeletal muscle, impairing glucose metabolism mediated by insulin in these tissues [10]. The increase in NEFAs can lead to hyperglycemia aggravation due to interactions of NEFAs with the insulin mediated glucose uptake. Under physiological conditions, skeletal muscle is the major consumer of glucose – around 90% while adipose tissue retains about 10%. However, in insulin resistant states, with high levels of plasma NEFAs, there is an accumulation of NEFAs, as triglycerides, in this tissue

[30], [31]. Furthermore, high plasma concentrations of NEFAs, also results in their accumulation in liver as triglycerides leading to two risky situations: hepatic steatosis and stimulation of gluconeogenesis that will increase plasma glucose levels [30], [32], [33]. In addition, adiponectin is a protein synthesized by adipose tissue and described as anti-inflammatory, antidiabetic and with antiatherogenic properties. This molecule is reported has being decreased in T2DM and in insulin resistance states and, more importantly. It has been pointed out as a good predictor for the development of hyperglycemia [34]–[36]. Adiponectin acts on liver, due to its anti-inflammatory and insulin-sensitizing capacity, decreasing fat depots in this tissue.

The postprandial glucose metabolism in T2DM patients is also impaired. In fact, under physiological conditions, endogenous glucose production is inhibited after food intake; however, in insulin resistant individuals, this mechanism is not totally inhibited [10]. Also, insulin resistance in the liver is the main factor that causes non-alcoholic fatty liver disease (NAFLD), which is also associated to impaired gluconeogenesis and is also known that accumulation of lipids, that could be released by adipose tissue in lipolysis, on the liver leads to a decrease in insulin sensivity [37]. Insulin resistance in liver is also characterized by an impaired inhibition, by insulin, of very low density lipoprotein production that leads to hypertriglyceridemia [10].

5. Prediabetes, impaired fasting glucose and impaired glucose tolerance

The term prediabetes has been largely nonconsensual. In 1980, the World Health Organization (WHO), rejected the term pointing to the fact that

having high risk factors such as increased glucose levels was no reason for alarm because it may not necessarily progress to the diabetic state, and this discussion still persists nowadays. On the other hand, the ADA, in 2005, returned with the term, using it to define individuals with impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), however, other risk factors were not considered at that time by ADA [38]. Later, in 2008, WHO suggested another equivalent term for prediabetes: intermediate hyperglycemia to define IFG and IGT. However, presently the ADA is not only taking into account the IFG and IGT terms but, it has added yet another condition, glycated hemoglobin (HbA1c) [38]–[40].

Thus, prediabetes or intermediate hyperglycemia is a high-risk state for the development of diabetes and they can be diagnosed through screening for IFG, IGT and HbA1c values. The fasting glucose levels should be evaluated after an overnight of at least 8 hours; moreover, patients should avoid factors that can alter carbohydrate metabolism, like exercise and the consumption of caffeine. The fasting glucose levels that are indicative of the prediabetic state are in the range of 100 - 125 mg/dL. In addition, the plasma glucose levels should be registered 2 hours after a 75g oral glucose tolerance test (OGTT), taken in the morning also after an overnight fast and the respective values should be between 140–199 mg/dL [40]. People with IGT have 60% of risk for developing diabetes within ten years and also 50% chance for coronary heart disease [41].

The HbA1c can be a marker for diabetes risk as well as microvascular complications. Already in 1993 the Diabetes Control and Complications Trial stated the importance of HbA1c, but only more recently in 2009 it was

recommended as a diagnosis for diabetes by the International Expert Committee [42], [43]. Diabetes complications are more likely to appear in patients with HbA1c ≥ 6.5%, the critical value indicating the presence of diabetes. Patients with HbA1c between 6%-6.4% are at higher risk of developing diabetes [42], [44]. This test evaluates the hemoglobin levels present in blood within the past 1-3 months [45], by measuring the concentration of hemoglobin molecules that are attached to glucose, presented in percentage [42].

Evaluation of HbA1c levels should be performed under a method certified by the National Glycohemoglobin Standardization Program (NGSP) and standardized to the Diabetes Control and Complication Trial (DCCT) assay. The values of HbA1c indicative of a prediabetic state are normally within the range of 5.7% and 6.4%, as previously stated [38]–[40], [46].

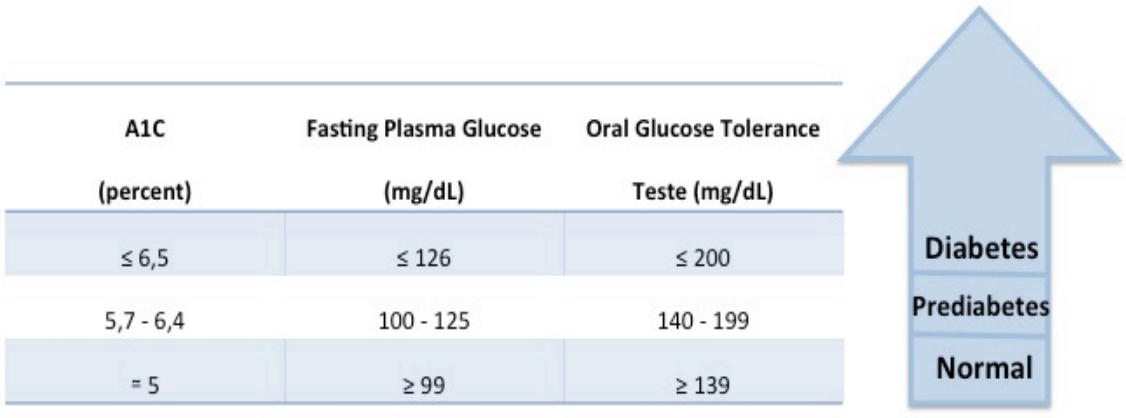

Figure 6. Blood test levels for diagnosis of diabetes and prediabetes.

(Adapted from National Diabetes Information Clearinghouse, http://www.niddk.nih.gov/Pages/default.aspx).

A gradual increase in these values is indicative of an increased risk of developing diabetes; however the risk increases in proportion of the quantity

of altered tests. Which means that the risk is greater if, alterations are detected in several tests

The discussion around the prediabetes term and condition is due to the unpredictable character of its progression to diabetes. This condition does not represent an actual disease state; just an elevated risk for diabetes and therefore it does not imply a treatment. In addition, despite the high risk, the disease may never reveal itself in some of the people while in others it may progress to the diabetic state. Furthermore, the risk of prediabetic individuals becoming diabetic is quite comparable to the risk that other individuals have to develop diabetes, without this condition but having other risk factors, such as family history, cardiovascular diseases, obesity, hypertension, history of gestational diabetes mellitus history, polycystic ovary syndrome and others [38], [47], [48].

There are studies that focus on risk of progression from the prediabetic to the diabetic state and, despite the distinct results – probably due to the population under study, the difference in age of the groups studied or the obesity level, for example – in general, it has been observed that about 25% of prediabetic individuals with either IFG or IGT evolve to T2DM in a time range of 5 years, 50% maintain the prediabetic condition and only 25% revert to normal and healthy values [25].

Studies aiming at preventing the progression of prediabetes to diabetes and its associated complications focus in lifestyle modifications, like weight loss, increase of physical activity, changes in diet and even pharmacological intervention with antidiabetic drugs had different approaches, varying in the alterations of lifestyle [39]. In some cases those alterations were

made individually, in others, in order to do a more complete intervention, were combined more factors, like diet change, increase physical activity and also the intake of antidiabetic drugs [25].

Studies lasting three years in which prediabetic individuals were followed with the aim of changing toward a healthier lifestyle and diet have shown positive results. Subjects achieved a decrease in 58% for the risk factors toward diabetes, and improved their insulin sensitivity and pancreatic beta-cell function [49].

It is estimated that in the USA prediabetes affects around 57 million people [39], while worldwide in 2010, only taking into account IGT and age ranges of 20-79, it was estimated that 344 million individuals were prediabetic [50]. Moreover, the prediction for 2030 is that there will be about 472 million prediabetic individuals, and according to ADA, 70% of the prediabetic people will develop T2DM [38], [47]. These values are alarming and it means that the prevalence of diabetes is greatly increasing. Therefore, it's crucial to develop early interventions for people with prediabetes, mainly by lifestyle alterations, in order to reverse the high diabetes risk (Figure 6.) [25].

6. Glucose metabolism

6.1 Insulin action

Insulin signaling begins with the secretion of the hormone by the pancreatic beta cells in response to high glucose levels, particularly after a meal. Next, insulin binds with high affinity to the insulin receptor (IR), and the receptor is autophosphorylated, recruiting and phosphorylating other

important cellular proteins as the insulin receptor substrate (IRS) family, in which IRS1 is one of the most important [10]. Previous studies have shown that T2DM and insulin resistance are associated with low cellular IRS1 [16], [18], [51]. IRS1 knockout homozygous animal models showed, hyperinsulinemia when compared with IRS1 heterozygous knockout and wild type animals, although no alterations in blood glucose levels. The phenotype of IRS1 whole body knockout mice seems to have some similarities with T2DM and the insulin resistant prediabetic state [52], [53]. The IR is inactivated by dephosphorylation by regulatory tyrosine phosphatases such as PTP1B (protein-tyrosine phosphatase 1B) leading to reduced activity/insulin signaling. Animal models with deficiency for PTP1B proteins have a higher insulin sensivity and have an improved insulin signaling and insulin sensivity *in vivo.* Also, whole body PTP1B knockout mice are also resistant to diet-induced obesity [54], [55]. All this associated modulation of insulin sensivity and obesity reduction suggests PTP1B a potential therapeutic target of T2DM [54]. PI3- Kinase (PI3K) has a regulatory (p85) and a catalytic (p110) subunits. Tyrosine phosphorylation of IRS1 leads to recognition of it from the regulatory subunit, p85, of PI3K, which, in turn will cause the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) by the catalytic subunit, a second messenger that will activate a serine/threonine phosphorylation cascade [56]. One of its targets is the Ser/Thr kinase PDK1, which phosphorylates and activates several down-stream kinases, including Akt/protein kinase-B (PKB) [55] (Figure. 7).

Akt/PKB is a serine/threonine kinase and its activation occurs through phosphorylation of Thr308 and Ser473. Active Akt/PKB mediates insulin

action by phosphorylation of substrates, such as kinases, signaling proteins and transcription factors. Some studies have reported a decreased insulin mediated phosphorylation of Ser473 or Thr308 in liver and skeletal muscle of patients with T2DM [57], [58] and insulin resistant states [59]. One of the Akt/PKB targets is GSK3 (Glycogen synthase kinase-3), a protein involved in glycogen synthesis. Akt/PKB plays an important role in the insulin-stimulated glucose uptake into adipocytes and muscle cells and this process seems to occur through phosphorylation and inhibition of the Rab-GTPase-activating protein, AS160 (for Akt substrate of 160 kDa). This will cause the activation of small Rab GTPases important for the cytoskeletal re-organization that is important for the translocation of glucose transporter 4 (GLUT4) to the plasma membrane, allowing for glucose uptake [58], [60] (Figure. 7). Studies have shown that phosphorylation of AS160 is reduced in patients with T2DM, leading to insulin resistance [60], [61].

Furthermore, Akt/PKB also phosphorylates and inactivates tuberin (tuberous sclerosis complex-2, TSC2) which complexes with hamartin (TSC1). This complex, TSC1/2, works as an inhibitor of the mammalian target of rapamycin (mTOR). Thus, when the Akt/PKB is active, it phosphorylates the TSC1/2 complex, no longer inhibiting mTOR (Figure. 7).

mTOR has the capacity to regulate protein and lipid synthesis by phosphorylating other proteins, such as p70 ribosomal protein S6 kinase (P70S6K) and eukaryotic translation initiation factor 4E binding protein-1 (EIF4EBP1) [55].

Moreover, Akt/PKB is also involved in the gluconeogenic process through mediated activation of the forkhead (FOX) class of transcription

factors, of which FOXO1 is one of them. FOXO1 will both activate gluconeogenic genes in liver and inactivate adipogenesis in adipose tissue, which is reversed by phosphorylation mediated by insulin [55] (Figure. 7).

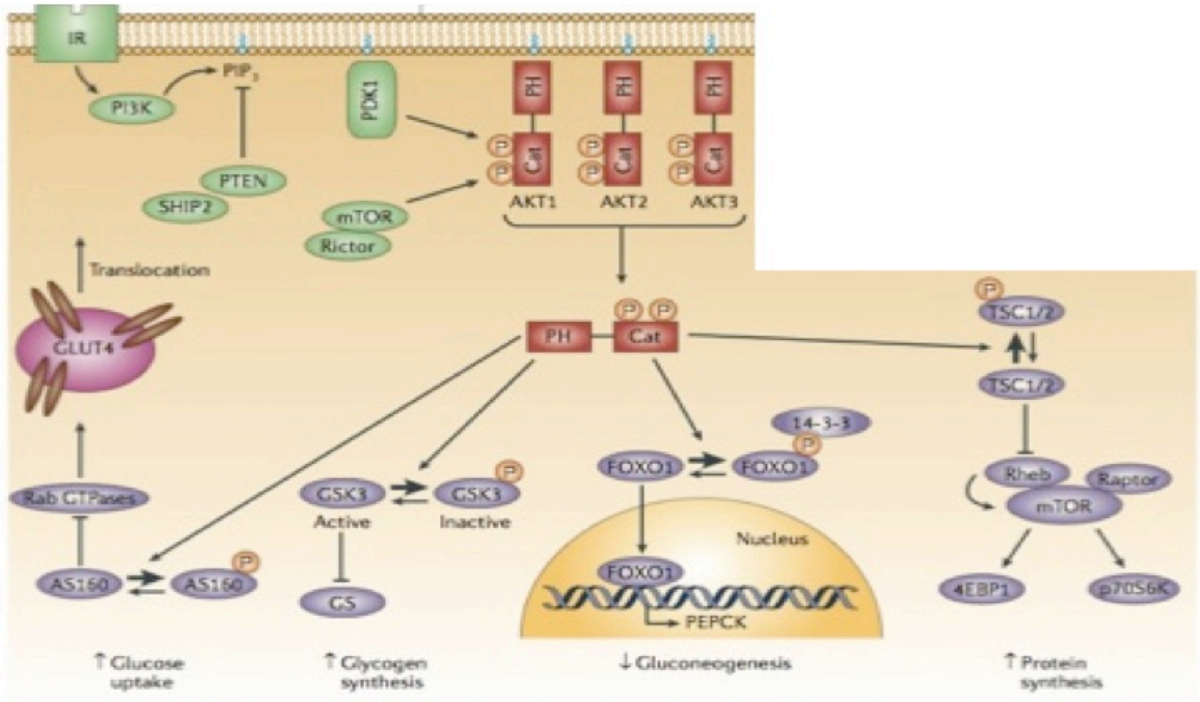

Figure 7. Insulin signaling pathway. Adapted from ref. [55].

6.2 Gluconeogenesis

Some tissues depend almost completely on glucose for their metabolic energy, as the case of the brain. During short periods of fasting, the liver produces and releases glucose mostly by glycogenolysis [37]. However, in some cases, as longer periods of fasting and in between meals or after vigorous exercise, the supply of glucose runs out and in these occasions, the organism needs to make glucose from noncarbohydrate molecules to maintain the blood glucose levels stable and avoid hypoglycemia [62].

Gluconeogenesis is the pathway by which new glucose is synthesized, and is a process usually mentioned as endogenous glucose production (EGP), since glucose is synthetized *de novo* by the liver (Figure. 8). In this process, new glucose molecules are produced from simple carbons as lactate, alanine and glycerol. Insulin suppresses gluconeogenesis, so, in case of low insulin states, as in the post-absorptive state, tissues don't take up glucose, and it is oxidized or suffers glycolysis to be converted into alanine and lactate that will be used by the liver for gluconeogenesis [10], [63]. Lactate is converted to pyruvate by lactate dehydrogenase and it is transformed into oxaloacetate by pyruvate carboxylase inside the mitochondria. Then oxaloacetate is reduced to malate by mitochondrial malate dehydrogenase and it goes to the cytoplasm to be oxidized by cytoplasmic malate dehydrogenase to regenerate oxaloacetate. In turn cytoplasmic oxaloacetate is converted to phosphoenolpyruvate by cytoplasmic phosphoenolpyruvate carboxylase (PEPCK-C), a key step of gluconeogenesis. This step is so important that is has been proven that deletion of PEPCK-C leads to death within 3 days after birth. Phosphoenolpyruvate is next converted into fructose 1,6-biphosphate (F1,6P) which is then dephosphorylated by fructose 1,6 bisphosphatase (FBPase) to generate fructose-6-phosphate (F6P). F6P is converted to glucose-6-phosphate (G6P), transported into the endoplasmatic reticulum (ER), and dephosphorylated by G6Pase to generate glucose [10], [37]. Mice with hepatocyte-specific deletion of G6Pase develop hyperlipidemia and hepatic steatosis [36].

FOXO1 is one of the transcription factors responsible for stimulating the expression of phosphoenolpyruvate carboxylase, G6Pase and peroxisome proliferator γ- activated receptor coactivator 1-α (PGC-1α). Studies have shown that FOXO1 knockout mice have decreased glycogenolysis and gluconeogenesis in the fasted state leading to low levels of plasma glucose [37].

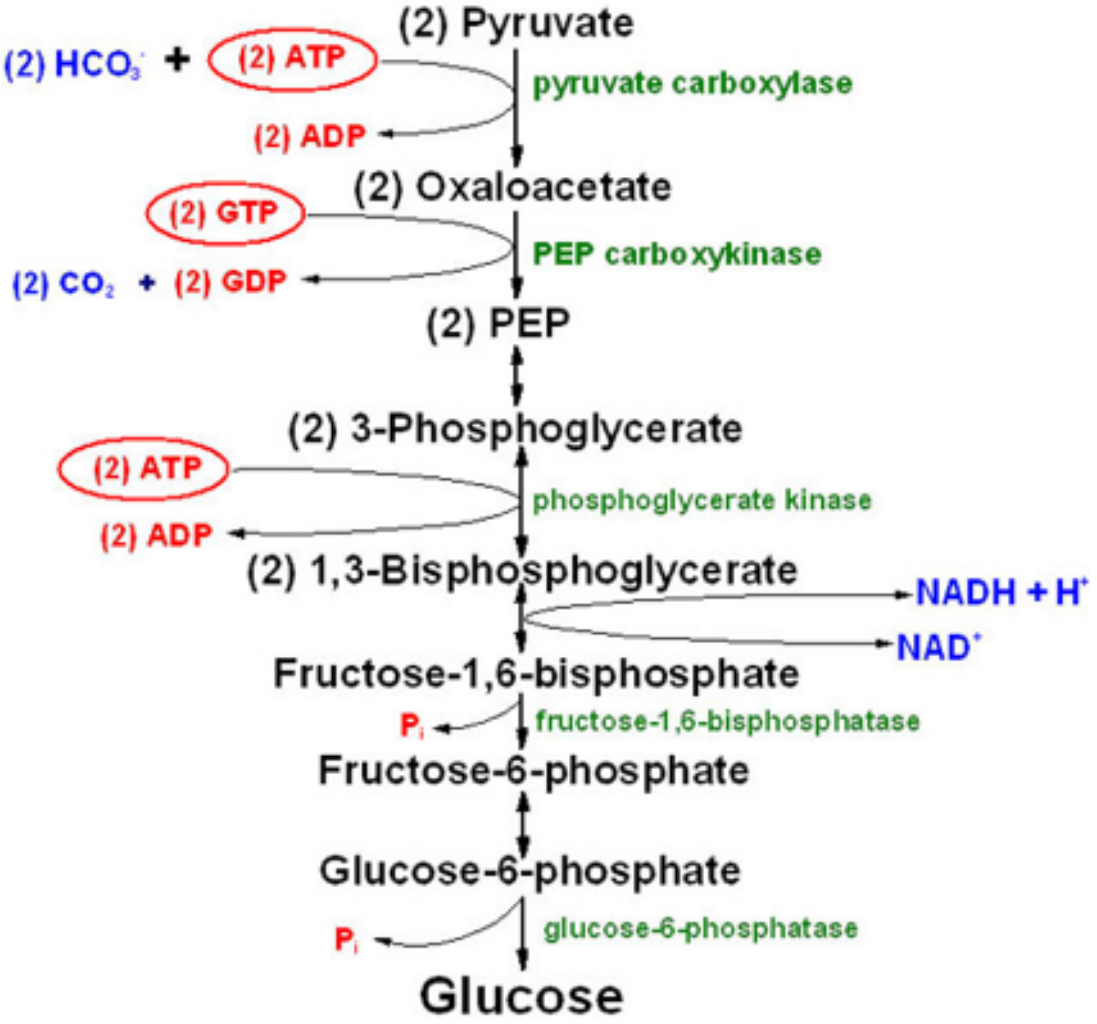

Figure 8. An overview of gluconeogenesis.

In diabetes, both in fasting as in the postprandial state, there is an increased endogenous glucose production, which contributes to the characteristic hyperglycemia [63], [64]. In addition, studies [65] have shown

that prediabetic subjects already have, abnormalities in glucose production resulting from an increased gluconeogenesis [10].

6.3 Glycogenesis and glycogenolysis

Insulin has the capacity to stimulate glucose uptake and storage it as glycogen in the fed periods – glycogenesis [10] (Figure. 9). Hepatic cells have GLUT2, a plasma membrane glucose transporter that allows glucose to enter into these cells to be phosphorylated by glucokinase (GK) and converted into glycogen by glycogen synthase (GS), decreasing plasma glucose levels. GS is activated by phosphorylation of Akt, which inactivates glycogen synthase kinase 3 (GSK-3) leading to an increased glycogen synthesis. Also, insulin restrains glycogenolysis by inhibiting glycogen phosphorylase and increasing levels of G6P [37], [62].

While in the fasted state, another mechanism occurs – glycogenolysis, which is the conversion of glycogen into glucose by glycogen phosphorylase (Figure. 9). Insulin levels are low and GS is inhibited unlike glycogen phosphorylase, which is activated in the fasted state [37], [54].

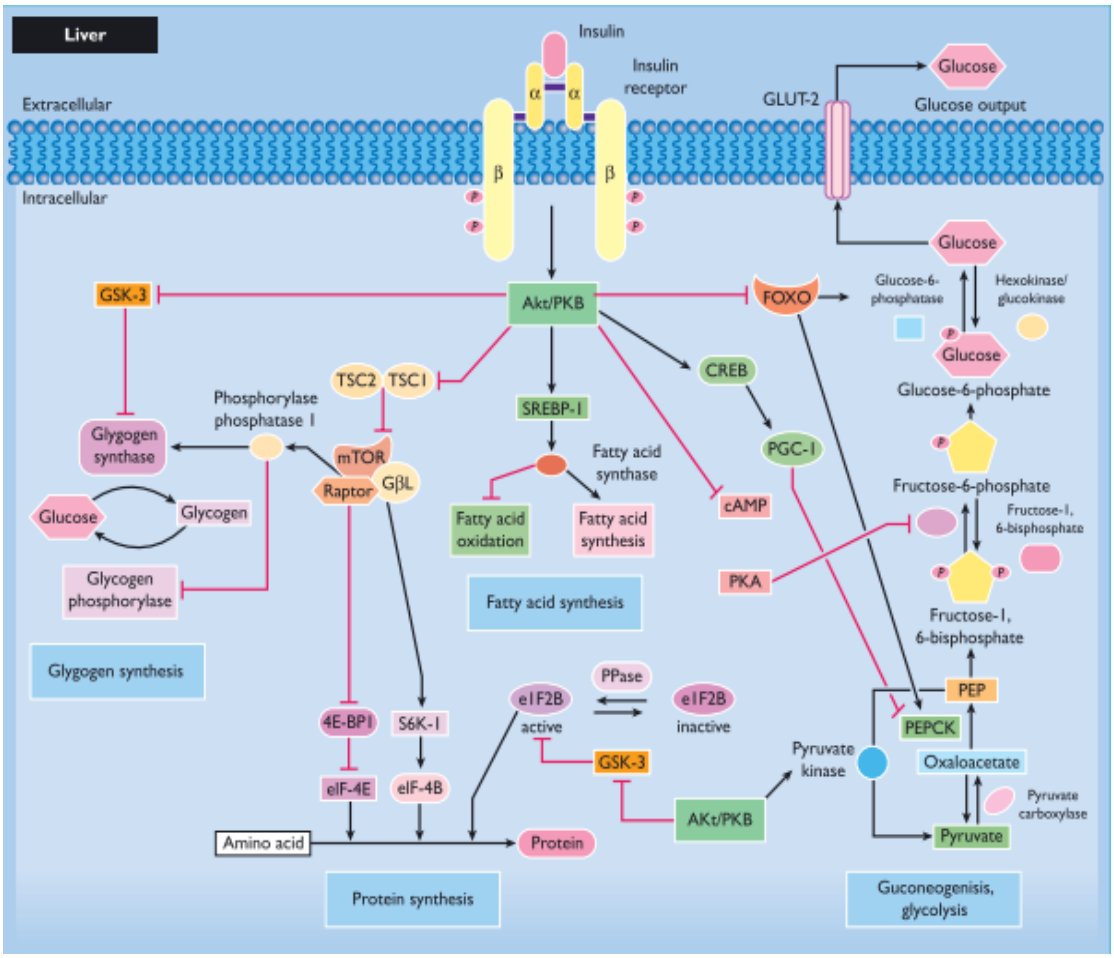

Figure 9. Insulin pathways involved in glycogen synthesis, gluconeogenesis and glycolysis, in the liver. Borrowed from ref. [10].

7. Lipid metabolism

7.1 Lipogenesis

The accumulation of fat in adipocytes is a mechanism that results from the balance between triglyceride synthesis (fatty acid uptake and lipogenesis) and breakdown (lipolysis/fatty acid oxidation). Lipogenesis is the process where glucose is transformed into fatty acids and triglycerides are synthetized (Figure. 10). Lipogenesis occurs in both adipose tissue and liver

trough stimulation of insulin [10], [32], [66]. Periods of fasting or excess of food intake modulates the expression levels of lipogenic genes, which can explain the low plasma levels of glucose and elevated levels of free fatty acids during fasting. Plasma glucose levels stimulate lipogenesis trough several processes. As mentioned before, glucose is involved in lipogenesis when it is transformed into acetyl-coA, promoting fatty acid synthesis. Furthermore, glucose stimulates lipogenesis by inducing the release of insulin and inhibiting the release of glucagon, a hormone released by pancreatic alpha-cells that opposes the action of insulin and stimulate glycogenolysis (degradation of glycogen), gluconeogenesis (glycerol conversion to glucose) and lipolysis (release of glycerol and fatty acids from triglycerides) [37], [67].

Insulin plays an important role in lipogenesis, increasing glucose uptake into adipose tissue, augmenting the disposal of glycerol and fatty acids that will be used for triglycerides synthesis. Besides this, insulin can also modulate important lipogenic and glycolytic enzymes by post-translational modifications and it regulates gene expression. Insulin activates, by phosphorylation, the Akt/PKB pathway by increasing lipogenesis and inducing the expression of FAS, an important enzyme involved in the *de novo* lipogenesis, as well as in the conversion of acetyl-coA and malonyl-coA into long-chain fatty acids, and sterol regulatory element-binding protein 1 (SREBP-1), which is a transcription factor that regulates genes involved in the synthesis and uptake of fatty acids and triglycerides [68]. SREBP-1 and ChREBP are regulators of *de novo* lipogenesis, intervening in the activity of many enzymes, such as acetyl-coA carboxylase (ACC) and FAS.

FAS is a key enzyme for lipogenesis responsible for the *de novo* synthesis of long chain saturated fatty acids [32]. While SREBP-1 is activated by insulin, ChREBP is activated by glucose. Thus, simultaneous states of hyperinsulinemia and hyperglicemia promote lipogenesis [66]. FAS activity is also regulated by peroxisome proliferator-activated receptor g coactivator 1 α (PGC-1α) which promotes lipid oxidation and increases lipogenesis in muscle [66] [69]. Studies have shown that PGC-1α is decreased in insulin resistant states and that is also involved in obesity [70].

CD36 plays an important role in regulating cellular uptake of free fatty acids that will lead to storage and release of triglycerides in adipose tissue [66], [71]. However, when the amount of FFA exceeds the storage capacity of adipose tissue, these FFA are transported to other tissues, such as skeletal muscle and liver, resulting in decreased insulin sensitivity in those tissues [71]. Previous studies with animal models of hyperinsulinemia reported an increase of hepatic CD36 expression correlated to hepatic steatosis and insulin resistance [72].

Moreover, another important transcription factor in adipose tissue is the family of peroxisome proliferator-activated receptors (PPAR). They have important roles in the regulation of glucose levels, fatty acid and lipoprotein metabolism, cell proliferation, differentiation and inflammation. PPARα is expressed in tissues with high levels of fatty acid catabolism. It regulates the transcription of genes involved in glucose metabolism in many tissues, such as the liver and skeletal muscle [73]. PPARδ, also known as PPARβ, is not only very expressed in the liver and skeletal muscle but also in adipose tissue. Activation of this receptor in the liver seems to decrease hepatic glucose

output contributing to glucose tolerance and improvement of insulin sensitivity. Recent studies have shown that knockout mice for PPARβ have glucose intolerance and treatments with PPARβ agonist have decreased free fatty acids and improved insulin sensitivity in mice and in moderately obese men [73].

Peroxisome proliferator-activated receptor gamma (PPARg) is activated by fatty acids and is involved in the maturation of pre-adipocytes into mature fat cells – adipogenesis – and also in lipogenesis. Studies have shown that dysregulation on this molecule leads to alterations in lipid storage and mobilization, the main problem of obesity [32]. In postprandial states, PPAR g is highly expressed and its activation modulates genes that mediate fatty acids uptake and storage. Subjects with metabolic alterations, such as insulin resistance and obesity, have decreased PPAR g levels in both fasting and postprandial periods.

Diacylglycerol acyltransferase 1 (DGAT1) is an enzyme that catalyzes the final step of triglycerides formation. Previous studies reported that DGAT1 knockout mice, although viable, have a decrease in triglyceride synthesis and an increase in insulin sensitivity [74].

7.2 Lipolysis

Lipolysis equilibrates the metabolic fuels, such as glucose and free fatty acids according to the energy needs of the cell and insulin has a crucial role on it due to its antilipolytic action. When an organism goes through fasting periods, the liver and skeletal muscle use FFAs as fuel and convert it into

ketones bodies that will replace the glucose needed by the nervous system. Adipose tissue produces and releases NEFAs and glycerol from stored triglycerides and this conversion is done by hormone-sensitive lipase (HSL) [30], [37]. Instead, during feeding, this mechanism is decreased and triglycerides are stored [10], [37], [75].

Lipolysis is impaired in states of insulin resistance and in T2DM due to in part the lack or ability of insulin to stimulate glucose uptake into target tissues, such as skeletal muscle and adipose tissue, and effectively inhibit lipolysis in fat depots, leading to increased fatty acids in circulation [33].

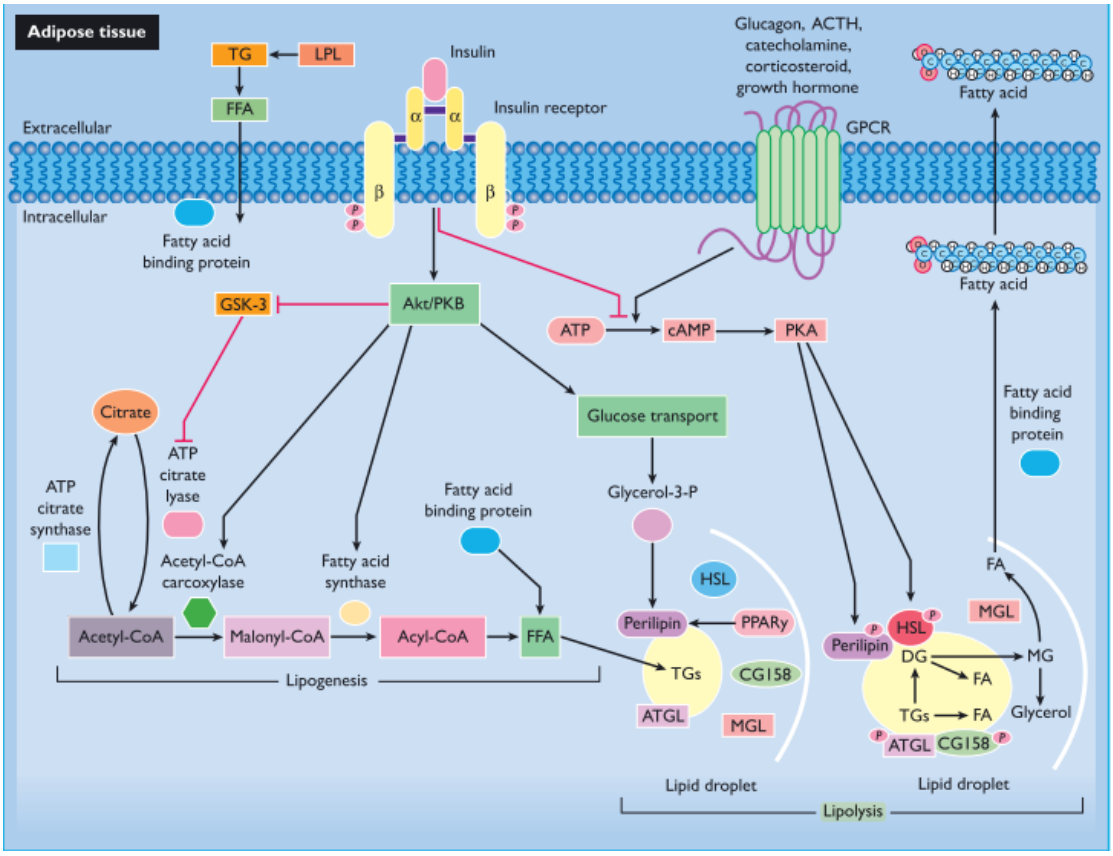

Figure 10. Insulin action in lipolysis and lipogenesis. Borrowed from ref. [10].

8. The impact of western diet on insulin resistance and prediabetes

Going back to the late seventies, when the low-fat diet was recommended to American citizens it has been possible to see a massive increase in obesity and diabetes, nearly doubling their numbers, until now (Figure 11 and 12.) [76], [77].

Nowadays, a low-fat, high carbohydrate diet in association with exercise is still the recommendation for a healthy lifestyle. However, rich carbohydrate diets are associated with several health problems as postprandial plasma glucose and insulin secretion, thereby increasing risk of CVD (cardiovascular disease), hypertension, dyslipidemia, obesity and diabetes [78], [79].

However, not all carbohydrates are identical. They're classified as simple or complex, according to their chemical structure. The complex carbohydrates are the most recommended for a good diet, on the other hand, simple carbohydrates should be avoided, because as they are absorbed more quickly cause a more rapid postprandial glucose response [80].

It is interesting to see that this obesity and diabetes epidemic started around the time that those guidelines were recommended [76], [77], when people started to leave the traditional food, high in fat, like butter and lard and began to consume processed food low in fat but with high percentage of simple sugar [81].

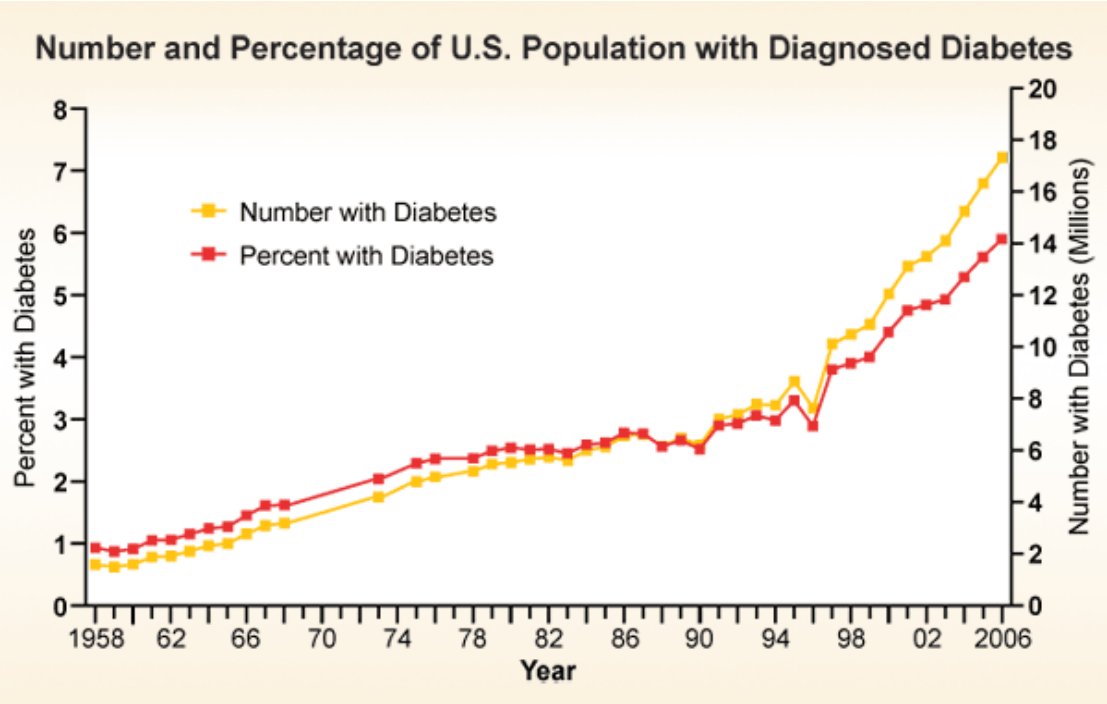

Figure 11. Diabetes progression over the last 50 years.
(http://drtouchinsky.com/2010/02/25/health-stats-and-trends-of-our-united-states-of-america/)

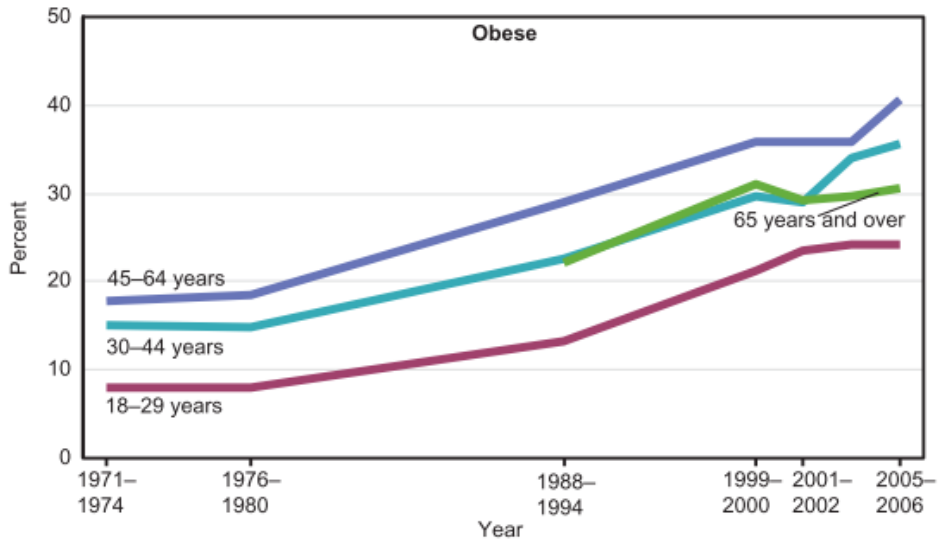

Figure 12. Obesity epidemic since the seventies. Borrowed from ref. [76].

Many studies have shown that low fat diets are neither efficient on weight loss nor on health improvement when compared with low carbohydrate diet [82]–[85], showing that low carbohydrate diets have greater improvements on insulin sensivity and triglycerides levels [86] even showing good results on reversing T2DM and the control of glycemic levels [87]. In addition, it has been reported that western diets, moderate in fat cause insulin resistance and body weight increase of about 60% [88], [89]. Moreover, other studies comparing a mediterranean diet, based on the consumption of minimally processed foods, high consumption of vegetables, fruits, unrefined grains, fish, vegetable proteins, vegetable fats mainly from olive oil, moderate consumption of red wine, and western diet, rich in meat, processed foods, and sweets, have shown that the mediterranean diet could have a protective role regarding the metabolic syndrome [90]. The molecular mechanisms by which simple sugars cause insulin resistance and prediabetes is still not very well understood. Therefore we sought to study this phenomenon in an animal model of prediabetes.

9. Aim of the study

In light of the above and taking into account the high prevalence of insulin resistance and prediabetes in our society, pivotal pathological states that precede diabetes but that are still poorly understood, we sought to evaluate some of the mechanisms underlying the development of insulin resistance in insulin responsive tissues. Using an insulin resistant and

prediabetic rat model, we focused our studies understanding glucose and lipid metabolism, in adipose tissue, liver and skeletal muscle.

II. Materials & methods

II. MATERIALS & METHODS

1. Animal model and diet

Adult (16 weeks old) male Wistar rats (Charles River Laboratories, Barcelona, Spain) were used for our study. Animals were housed in the animal facility of the Laboratory of Pharmacology and Experimental Therapeutics (IBILI, Faculty of Medicine, University of Coimbra) and kept at a constant temperature (22-23°C) and light (12:12-h light-dark cycle).

After 1 week of acclimatization, animals were randomly divided into two groups: control group (n=12) and the HSu group (n=10). All the rats have received standard rat chow (containing 16.1% protein; 3.1% lipids; 3.9% fibers and 5.1% minerals, (AO4 Panlab, Barcelona, Spain) and water *ad libitum*. During 9 weeks of treatment, the control group received tap water and the HSu group received 35% sucrose (S0389; Sigma-Aldrich) in the drinking water. The body weight (BW) of animals and the amount of ingested chow were registered weekly (on mondays) during the 9 weeks of treatment, using an analytical balance (KERN CB 6 K1, Germany). The volume of water and sucrose solution ingested by animals was controlled three times a week (on mondays, wednesdays and fridays). All experiments were conducted according to the National and European Directives on Animal Care, as well as, to the local ethics authorities.

2. Glucose tolerance test

After a fasting period of 6h rats were given an intraperitoneal (i.p.) injection

with a glucose bolus of 2g/kg of a glucose solution of 0.6 g/ml. The blood glucose levels were measured from the tail vein and the samples were taken before (0 min) the bolus and 15, 30, 60, and 120 min after the glucose injection using a glucometer (AccuChek Active, Roche Diagnostics Inc., Indianapolis, IN, USA) [91].

The area under the curve (AUC) for the GTT was calculated through the trapezoidal method [92].

3. Insulin tolerance test

This test was performed after a 6h, food removal and blood glucose levels were measured after an i.p injection of insulin (Novo Nordisk, Lisbon), 0.75 U/kg. Glucose values were evaluated from the tail vein blood and taken immediately before (0 min) and after 15, 30, 45, 60 min of the i.p administration of insulin. The blood glucose levels were evaluated with a glucometer (AccuChek Active, Roche Diagnostics Inc., Indianapolis, IN, USA) [91].

4. Sacrifice and tissue samples collection

At the end of treatment (week 9) and after an overnight fasting period, rats BW was measured and control and HSu animals were subdivided in two groups. One subgroup (Control – n=6; HSu-treated rats – n=5) received an i.p. insulin bolus (10 U/kg) and 10 min after the insulin bolus, animals were sacrifice by cervical dislocation. The other subgroup (Control – n=6; HSu-treated rats – n=5) received an i.p. saline injection. The fasting glycemia levels were evaluated by venipuncture from the jugular vein and measured with a glucometer (AccuChek Active, Roche Diagnostics Inc., Indianapolis, IN, USA). Epididimal adipose tissue, skeletal muscle

and liver samples were immediately removed, frozen in dry ice and stored at -80 for gene expression and Western-blot (WB) analysis.

5. Nonesterified fatty acids quantification

The blood collected at the sacrifice was used to evaluate serum non-esterified fatty acids (NEFA) using an FFA kit (NEFA C-test Wako, Wako Pure Chemicals, Neuss, Germany).

6. Western blot analysis

With this technique, we can separate and identify specific proteins present in cell lysates. Western blotting comprises mainly three phases:

1. Electrophoresis in polyacrylamide gels: in this step separation of proteins occurs that involves their migration in a gel during an application of voltage. Proteins are separated according to their molecular weight;
2. Blotting: After protein separation on the gel, proteins are transfered from the gel to a polyvinylidene fluoride (PVDF) membrane. To complete this transfer an electrical field is used as in the electrophoresis. After blotting the membrane, it will be used to perform an immunoenzymatic assay (immunoblot);
3. Protein detection: To proceed with the protein detection, the membrane is blocked with a solution of 5% of milk diluted in Tris buffer (50 mM Tris.HCl, pH 7.4, 150 mM NaCl) with 0.01 % of Tween 20 (TBS-T, pH

7.4) and then incubated with the specific antibodies to the protein of interest. The primary antibody will specifically bind to the protein of interest and then the membrane is incubated with a secondary antibody that will bind the primary antibody producing proportional protein quantity fluorescence when exposed to a chemioluminescent agent. [93]

6.1 Cell lysate preparation

The samples were weighted according to the table X and homogenized in 550 µl of ice-cold RIPA buffer (20 mM Tris HCl pH 7.4, 25 mM NaCl, 1% NP-40 (Nonidet P-40), 5 mM EDTA, 10 mM Sodium diphosphate (Na4P2O7), 10 mM Sodium Fluoride (NaF), 2 mM Sodium Vanadate Na3VO4, 10 µg ml-1 Aprotinin from bovine lung, 1 mM Benzamidine and 1 mM Phenylmethylsulfonyl fluoride (PMSF).

Table 1. Weight of tissue samples used to perform cell lysates.

Sample	Weight
Epididimal adipose tissue	200mg
Liver	25mg
Skeletal muscle	50mg

Cell lysates were homogenized three times, during 10 seconds, with 5

seconds of interval, with an ULTRA-TURRAX® T 25 basic, IKA®-Werke (Staufen, Germany) homogenizer, to disrupt cells. After that, homogenized cells were placed on ice for about 30 min and, then, were centrifuged at 17 000 rotations per minute (rpm) at 4°C for 10 min. After this centrifugation, the supernatant was collected and it was again centrifuged at 17 000 rotations per minute (rpm) at 4°C for 10 min. After this second centrifugation, the lower phase was collected.

The protein concentration was determined using the bicinchoninic acid (BCA) method. After stored at -80ºC, cell lysates were denatured at 95ºC, for 5 min, in sample buffer (Tris HCl 0,5 M 0,4% SDS (pH 6,8); 0,6 M DTT; 30% (v/v) glycerol, 10% SDS (w/v) and 0.01% bromophenol blue).

6.2 SDS-PAGE, PVDF transfer and WB analysis

Depending on the protein of interest, 20, 40, or 60 μg of protein were were loaded in the gels. The electrophoresis was run on a 7,5% (v/v) sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with TBS-(50 mM Tris.HCl, pH 7.4, 150 mM NaCl) with 0.01 % of Tween 20 (TBS-T, pH 7.4) containing 5% dry milk for 1h at room temperature. Then, membranes were incubated overnight at 4ºC with a primary antibody dilution previously optimized by the group (table x). Mouse anti-β-actin (Sigma-Aldrich, A5316) and goat anti-actin (Santa Cruz Biotechnology) antibodies were used as loading controls.

After overnight primary antibody incubation, membranes were washed three times (five minutes each) with 0.01% TBS-T and incubated for 1h at room temperature with alkaline phosphatase-conjugated anti-rabbit antibody (1:5000) or

with alkaline phosphatase-conjugated anti-mouse antibody (1:10 000), depending on the origin of the primary antibody. After 1hour incubation, membranes were washed three times (five minutes each) with 0.01% TBS-T and expose to the ECF reagent, followed by scanning on a VersaDocTM Imaging System, Bio-Rad (Bio- Rad Laboratories, Amadora, Portugal). The generated signals were quantified using Quantity OneTM Software.

Table 2. List of antibodies used for Western blot, dilution and source.

Antibody	Dilution	Company
SREBP	1:1000	Santa Cruz
ChREBP	1:500	Santa Cruz
ACC1	1:1000	Cell Signaling
FAS	1:1000	Cell Signaling
DGAT1	1:200	Santa Cruz
PPAR α	1:1000	Santa Cruz
PPAR β	1:1000	Santa Cruz
PPAR γ	1:1000	Santa Cruz
FOXO1	1:1000	Cell Signaling
PGC1-α	1:750	Santa Cruz
PTP1B	1:250	Calbiochem
IRS-1	1:750	Santa Cruz
IRS-1 Tyr612	1:500	Invitrogen
PI3K85	1:5000	Millipore
Akt ser473	1:500	Cell Signaling
Akt Thr308	1:500	Santa Cruz
Akt	1:1000	Cell Signaling
AS160 Ser642	1:500	Cell Signaling
AS160	1:500	Cell Signaling
P70S6K	1:1000	Cell Signaling
P70S6K	1:1000	Cell Signaling
GLUT1	1:1000	Millipore
GLUT4	1:500	Millipore
mTOR Ser2448	1:500	Millipore
mTOR	1:1000	Cell Signaling
β**-actin**	1:5000	Sigma-Aldrich
Actin	1:1000	Santa Cruz

7. Statistical analysis

Results were analyzed as mean ± standard error of the mean (SEM) using GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA). Data with normal distribution were analyzed by parametric student's t-test. Non-parametric Mann Whitney test was performed to analyze data without normal distribution. Differences were considered significant when * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ or **** $p \leq 0.0001$.

III. Results

III. RESULTS

1. Metabolic characteristics of animals after chronic intervention with HSu diet

Body weight (BW) of the experimental animals was measured every week along the nine weeks of treatment. Control and HSu treated groups had a similar increase in body weight (386.9 ± 11.53 *vs* 390.4 ± 13.63 g) over the time measured (Figure 13 A). The volume of regular water (control group) and 35% sucrose enriched water (HSu group) consumed was quantified three times per week throughout the nine weeks of treatment. The volume of sucrose rich water consumed was significantly higher compared to the regular water consumed by the control group (120.3 ± 36.38 vs 90.39 ± 22.47 ml, $p < 0.01$) (Figure 13 B). In addition, the amount of food was also monitored weekly throughout the treatment period and results demonstrate that the control group had significantly higher food consumption compared to the HSu treated group (160.6 ± 16.31 *vs* 78.84 ± 22.21 g, $p < 0.01$) (Figure 13 C).

At the end of the 9 weeks of treatment, a glucose tolerance test (GTT) was performed, blood glucose levels where measured after an overnight fast at baseline and after an i.p. injection of glucose (2 g/kg BW) at 15, 30, 60 and 120 min in both groups (Figure 13 C). The HSu group presented significantly higher fasting blood glucose levels at baseline comparing to control animals (100.7 ± 3.315 *vs* 105.7 ± 0.9932 mg/dl), $p \leq 0.01$)

Maximum blood glucose levels in the HSu group were reached 30 minutes

after glucose injection, significantly different from the control group (222.3 ± 52.21 *vs* 424.0 ± 40.02, p ≤ 0.01 mg/dl). Sixty minutes after glucose injection, the HSu group persisted with significantly higher blood glucose levels (337.7 ± 46.42 *vs* 164.1 ± 25.09, p ≤ 0.01 mg/dl). Although not significantly different, 120 minutes after glucose injection, HSu treated animals still presented elevated levels of blood glucose, compared with control animals (138.3 ± 12.78 *vs* 230.9 ± 34.69, p = 0.0727). The glucose excursion observed for the HSu treated group was significantly slower compared to the control group. The area under the curve (40898 ± 7163 *vs* 79609 ± 8645, p ≤ 0.01 mg/dl) for the GTT was also significantly different, confirming impaired blood glucose tolerance (Figure 13 E).

Moreover, an insulin tolerance test (ITT) was also performed (Figure 13 F). Blood glucose levels were measured at baseline and after i.p. injection of insulin (0.75 U/kg BW) at 15, 30, 45, 60 and 120 min and the blood glucose values were significant higher in the HSu group at baseline (107.9 ± 1.402 *vs* 114.5 ± 0.9339 mg/dl, p < 0.01) and after 120 minutes of the insulin injection (42.60 ± 2.056 *vs* 73.20 ± 5.194 mg/dl, p < 0.01).

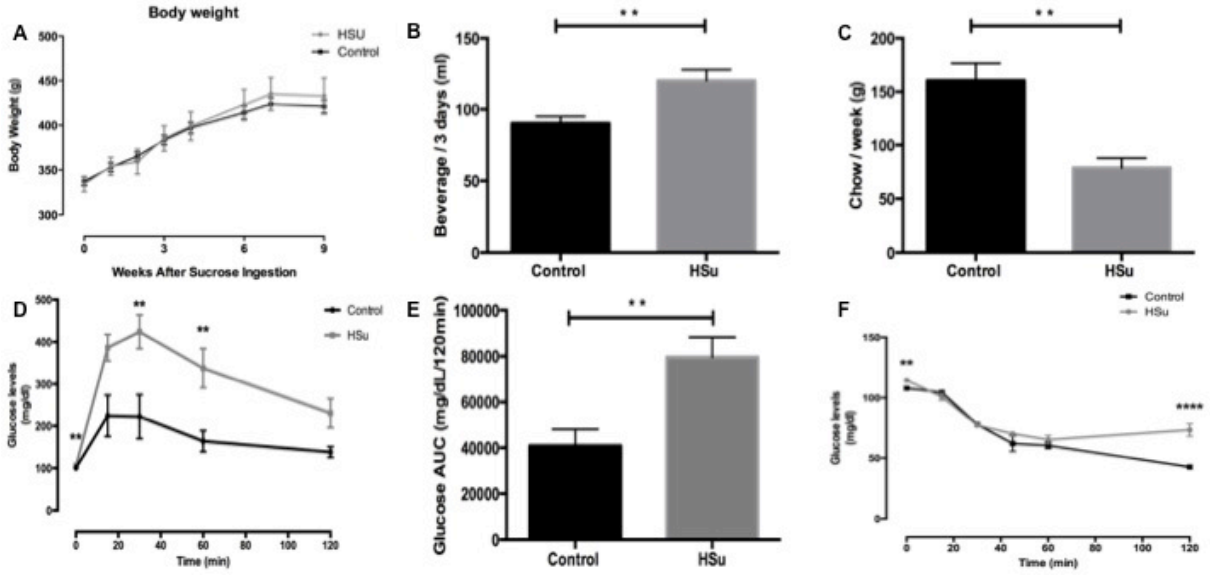

Figure 13. ***Metabolic characteristics of the animal model.*** Body weight, n=12 for Control and n=10 for HSu **(A)**; Drinking volume (per three days; mL) **(B)** and chow (per week; g) consumed **(C),** Glucose tolerance test (GTT) performed in the last week of treatment, n=7 per group **(D)**; Area under the curve (AUC) of total blood glucose after injection of glucose (2 g/kg BW) **(E)**; Insulin tolerance test (ITT) performed at the end of treatment, n=10 for Control and n=10 for HSu **(F)**. t-test., **p<0.01 and ****p ≤ 0.0001.

2. Glucose Metabolism

2.1 Insulin-stimulated glucose uptake in isolated adipocytes and glucose transporters in fat cells, liver and muscle after an intervention with HSu diet

We observed a significant increase in the insulin-stimulated glucose uptake in isolated adipocytes in both the control group (1.000 ± 0.1693 *vs* 1.899 ± 0.3161, p ≤ 0.05) and in the HSu treated group when compared to the basal in control mice (0.3663 ± 0.05822 *vs* ± 0.8888 ± 0.08739, p < 0.001). There is about a two-fold increase in glucose uptake induced by insulin in both groups when capered to basal. However, the relative insulin-stimulated glucose uptake in the HSu treated group was about half that observed in the control animals (1.899 ± 0.3161 vs 0.8888 ±

0.08739, $p < 0.01$). In addition, a significant difference was also observed between the basal glucose uptake of control compared to basal in the HSu treated group.

GLUT1 is the glucose transporter independent of insulin action always present in the plasma membrane in tissues. GLUT2 is characteristic of hepatocytes and GLUT4 is a glucose transporter that depends on insulin stimulation and is translocated from the intracellular vesicles to the plasma membrane in the presence of insulin and is very important in muscle and adipose tissue. Glucose transporter expression was studied in the three tissue types. We observed a significant increase of GLUT1 levels in the liver of control animals when compared with HSu treated animals (1.000 ± 0,1312 *vs* 0.4767 ± 0,08854, $p \leq 0.05$). We did not observe significant differences in the other glucose transporters analysed, either in muscle or adipose tissue.

An assay that would be interesting to complete in order to study cellular glucose transport would be to analyze if there is impairments on the translocation of GLUT4 from intracellular vesicles to plasma membrane in the presence of insulin, however, in the present study it wasn't possible to perform due to low amounts of tissue.

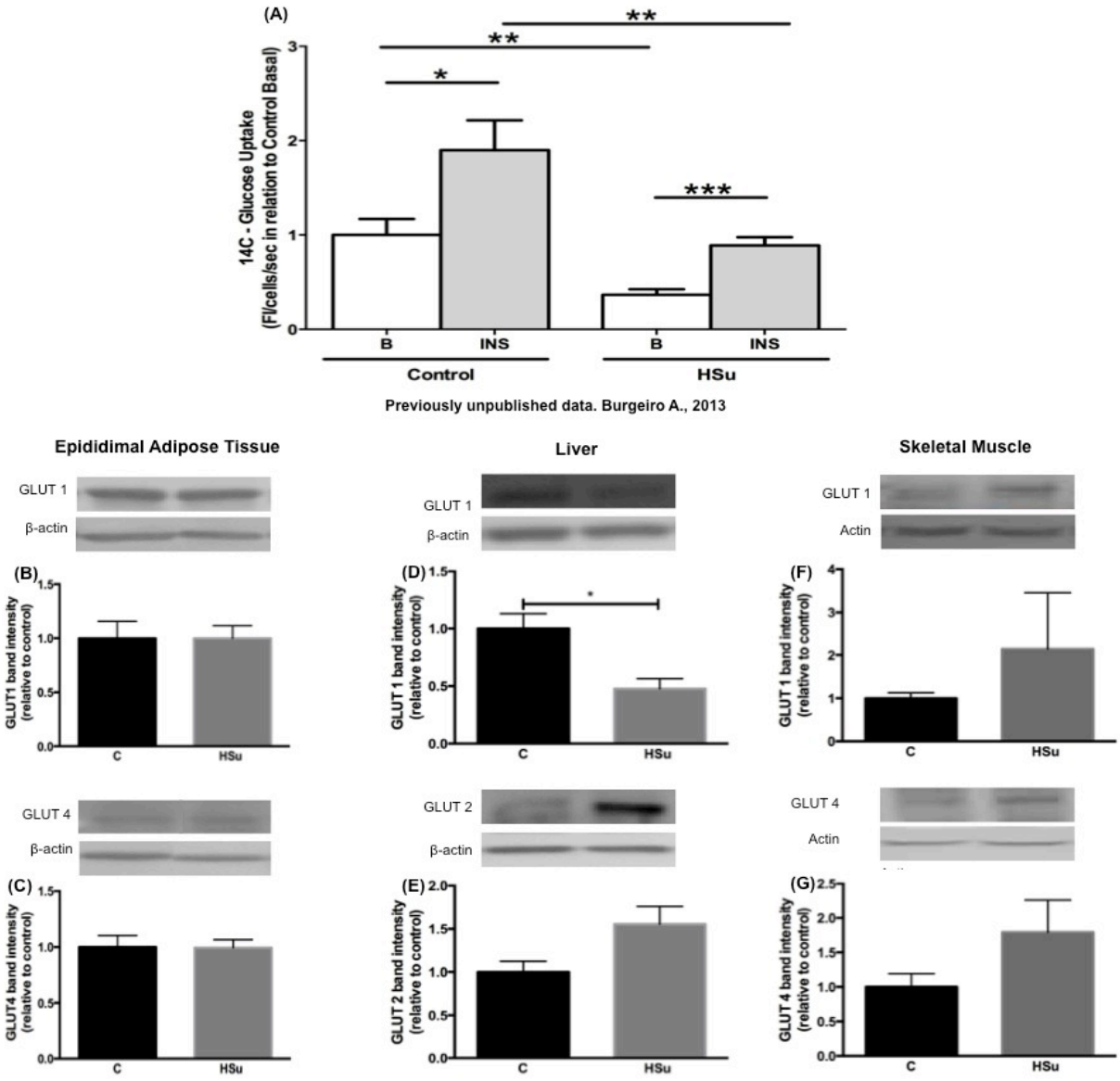

Figure 14. ***Effects of a high sucrose (HSu) diet on insulin stimulated glucose and glucose transporter expression.*** Glucose uptake in isolates adipocytes **(A)**, representative blots for protein expression of GLUT1 in adipose tissue **(B)** graphical representation of GLUT4 expression in adipose tissue **(C),** graphical representation of GLUT1 in liver **(D)**, graphical representation of GLUT2 in liver **(E),** graphical representation of GLUT1 in skeletal muscle **(F),** graphical representation of GLUT4 in skeletal muscle **(G).** Data are expressed as mean ± SEM, n=6 for Control groups and n=5 for HSu groups. C, Control; B, Basal; INS, stimulated with insulin. t-test, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.2 Modulation of critical nodes of the insulin signaling pathway in adipose tissue, liver and muscle by HSu diet

Several important nodes of the insulin signaling cascade were analyzed by Western Blot (Figure. 15). Phosphorylation of the insulin receptor substrate in Tyr 612 was studied, however, no significant differences were observed in either of the tissues analyzed (Figure. 15 A).

Akt/PKB is important in insulin action, it phosphorylates substrates, such as kinases, signaling proteins and transcription factors involved in the insulin signaling cascade. Therefore we measured its phosphorylation/activation by studying the two important residues needed for its activation, Thr 308 and Ser 473**,** however, under this treatment Akt/PKB signaling seems to remain intact in both groups, since the insulin mediated phosphorylation is similar in control and HSu groups. In addition, no significant differences were found in AS160 Thr 642 levels in either tissue (Figure 15 D.).

Moreover, we have measured mTOR, which intervenes on the insulin signaling cascade, no significant differences were observed in mTOR ser 2448 between the control and HSu groups for fat and liver, however there was a significant difference in the insulin stimulation of this residue in skeletal muscle in the HSu treated group. (6151 ± 0.1447 *vs* 1.335 ± 0.1311, ** $p \leq 0.01$) (control vs HSu) (Figure 15 E.).

Regarding P70S6k Thr 421/424, involved in regulation of lipid and protein synthesis, the phosphorylation observed in the presence of insulin was a significant difference in activation in isolated adipocytes in the HSu treated group (0.4495 ± 0.03192 *vs* 1.923 ± 0.3537, * $p \leq 0.05$)

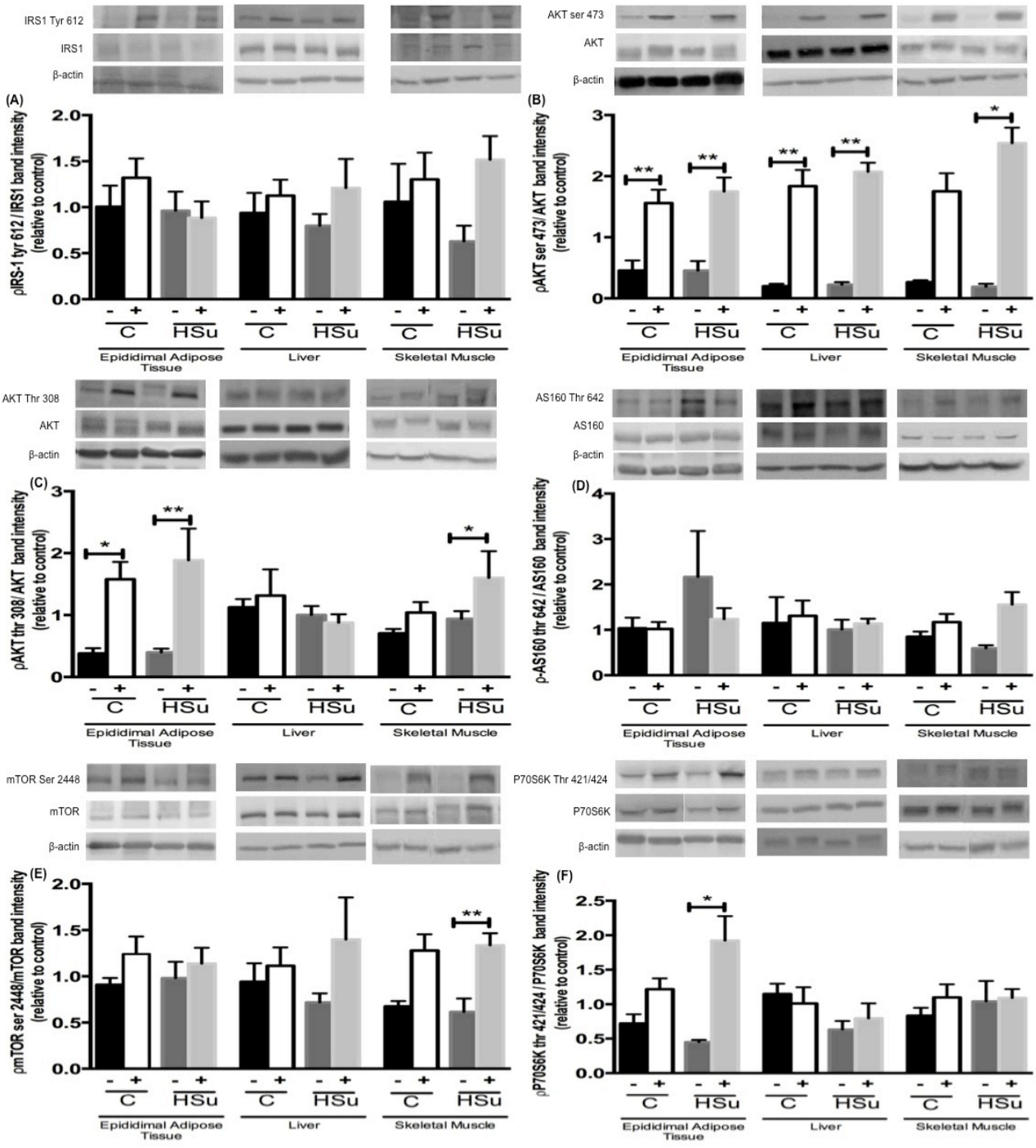

Figure 15. ***Effect of a diet enriched in sucrose (HSu) on important nodes of the insulin pathway.*** Isolated adipocytes, liver and skeletal muscle levels of IRS1 Tyr 612 **(A)**, Akt Ser 473 **(B)**, Akt Thr308 **(C)**, AS160 Thr 642 **(D)**, mTOR ser 2448 **(E)**, P70S6k Thr 421/424 **(F)**. Data are expressed as mean ± SEM, n=5/6 for control groups and n=4/5 for HSu groups. C, Control; -, without insulin stimulation; +, with insulin stimulation. t-test. * $p \leq 0.05$, ** $p \leq 0.01$.

3. NEFAs quantification and lipolysis assay

Serum non-esterified fatty acid quantification was performed after nine weeks of treatment in both the fasting and fed states. No significant differences in the total NEFAs concentration between the control and HSu groups were observed (Figure. 16). However with insulin stimulation, in the HSu group there was a decrease in NEFAs concentration (3.251 ± 0.1441 *vs 2.086 ± 0.09767,* $p < 0.001$*)* (Figure. 16 A). Moreover, even though basal lipolysis rates seem to be higher in the control group, the isoproterenol-stimulated lipolysis was highly increase (0.4495 ± 0.03192 *vs* 1.923 ± 0.3537) in the HSu group, while no significant decreases in lipolysis were observed in the presence of insulin (Figure 16. A).

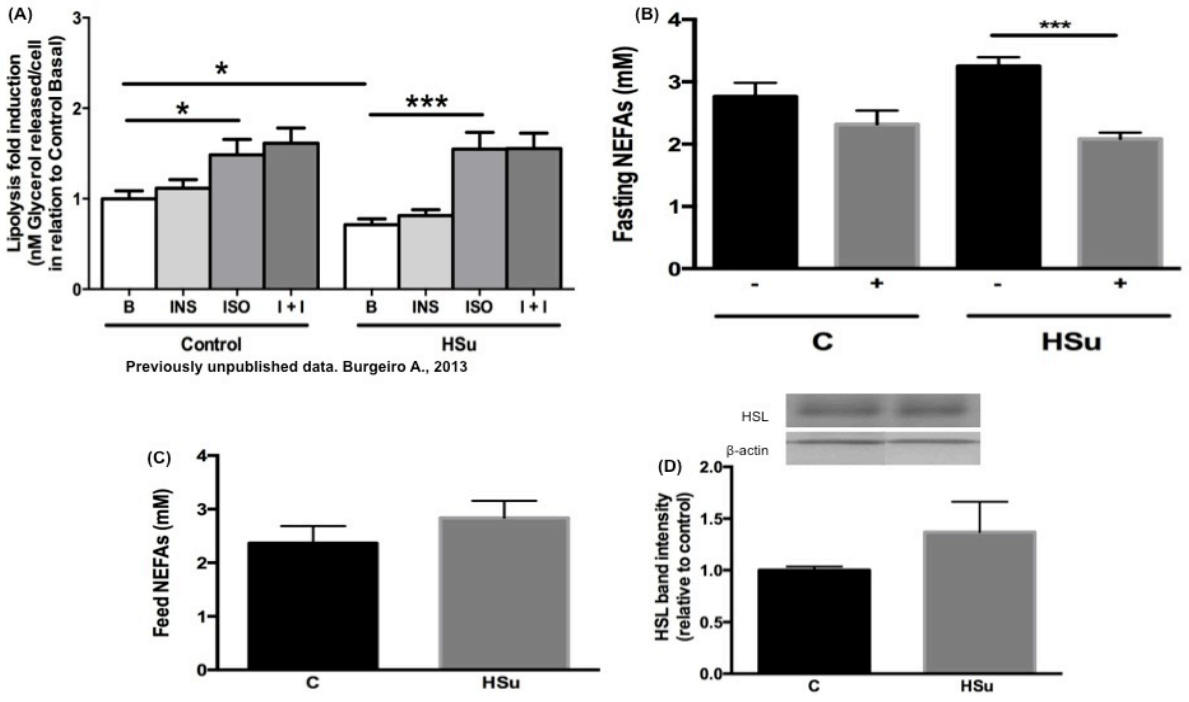

Figure 16. ***Lipolysis in isolated adipocytes (A), Fasting (***n=5 for c -; n=5 for c+ and n=6 for HSu -; n=6 for Hsu +) ***(A) and Fed (****n=7 for both control and HSu groups)* ***(B) NEFA levels in Control and HSu-treated group after 9 weeks of treatment.*** Data are expressed as mean ± SEM, C, Control; -, without insulin stimulation; +, with insulin stimulation; B, Basal; INS, stimulated with insulin; ISO, stimulated with isoproterenol; I+I, stimulated with both insulin and isoproterenol.

4. Are transcription factors important in glucose and lipid metabolism modulated by HSu diet?

The transcription factors SREBP and ChREBP, which are involved in fatty acids synthesis, were analyzed and a significant difference was registered in ChREBP expression in isolated adipocytes in the HSu group, compared to the control group (1.000 ± 0.07842 *vs* 1.276 ± 0.09476, $p \leq 0.05$) (Figure. 16 A/B). In the other tissues, no significant differences were observed. In addition, there was also a tendency for an increased expression of SREBP in liver, however given the small n no significant was obtained.

The transcription factor FOXO1, which is involved in gluconeogenic processes, revealed no significant differences between the control and HSu groups in the three tissues analyzed (Figure. 15 C).

PGC1-α was also assessed in order to analyze its involvement in lipogenesis and regulation of FAS, however no significant differences were observed in any of the groups studied under these conditions (Figure. 15 D).

In addition, PPAR-α, PPAR-β and PPAR-γ were analyzed in order to understand if there were alterations in lipid metabolism, however no significant differences were observed under these conditions and with the small n used for this study, however there is tendency for a decrease in PPAR-γ muscle.

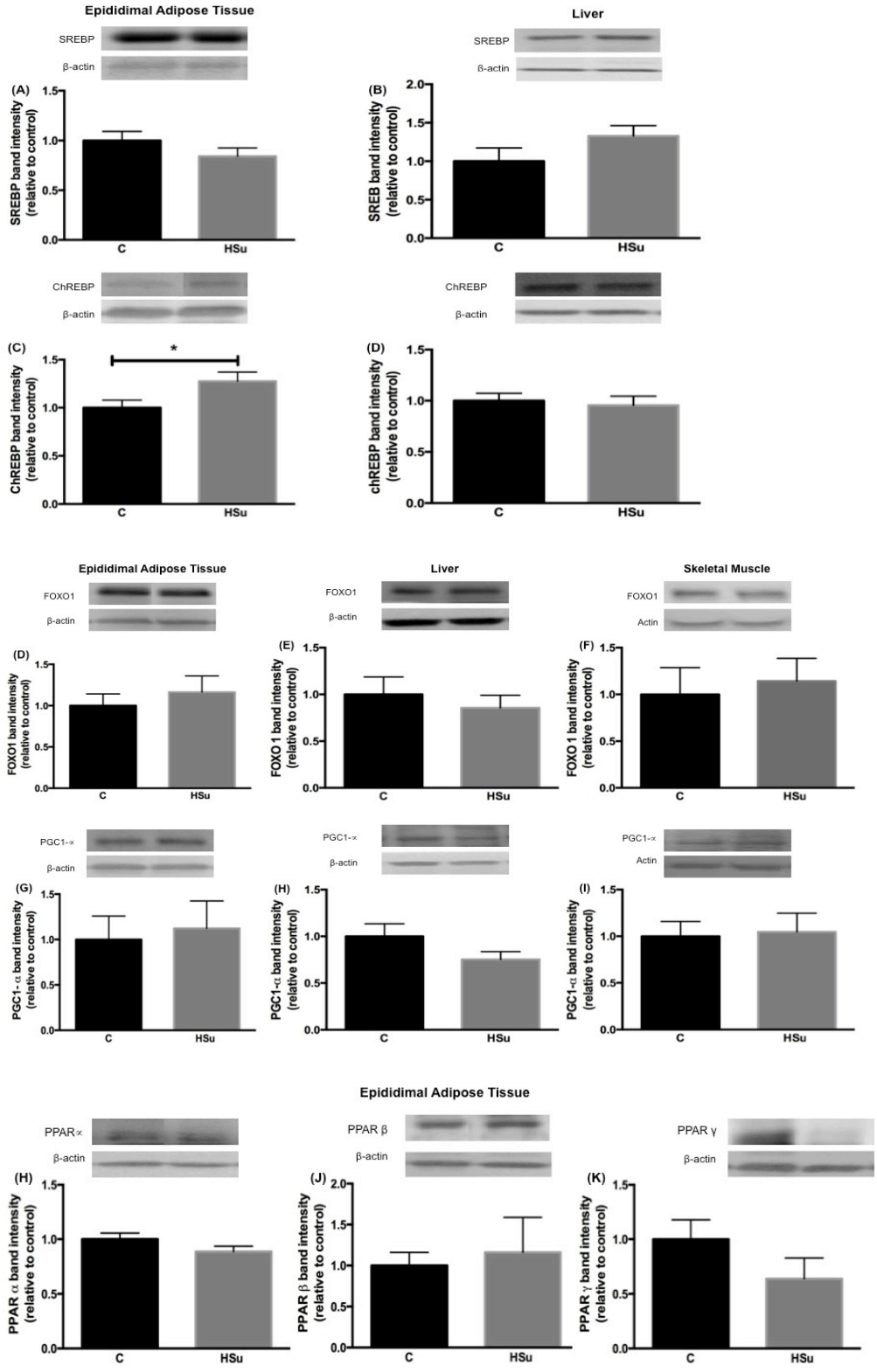

Figure 17. ***Expression of transcription factors involved in glucose and lipid metabolism after an intervention with HSu diet.*** SREBP **(A),** ChREBP **(B)**, FOXO1 **(C)**, PGC1-∝ **(D) and** PPAR family **(E)** levels. Data are expressed as mean ± SEM, n=6 for Control groups and n=5 for HSu groups. C, Control. t-test, * $p \leq 0.05$.

5. Lipogenic proteins in adipose tissue, liver and muscle

Since CD36 enhances cellular fatty acid (FA) uptake, a key step in energy metabolism, we have measure it; however, no differences were found in any group (Figure. 18 A). ACC1 have a key role in lipogenesis, therefore we measure ACC1 expression levels in both adipose tissue and liver. No significance was reached in this protein in both tissues (Figure. 18 B).

FAS protein is involved in synthesis of fatty acids, therefore its expression was also investigated and, while no significant differences in isolated adipocytes, in liver we observed a significant increase in FAS expression in the HSu group when compared to the control group (1.000 ± 0.1025 *vs* 2.349 ± 0.2097, $p \leq 0.05$) (Figure. 18 C).

Due to DGAT1's role in lipogenesis, we studied the expression levels of this protein; however, no significant differences were observed in this protein in any of the groups (Figure. 18 D).

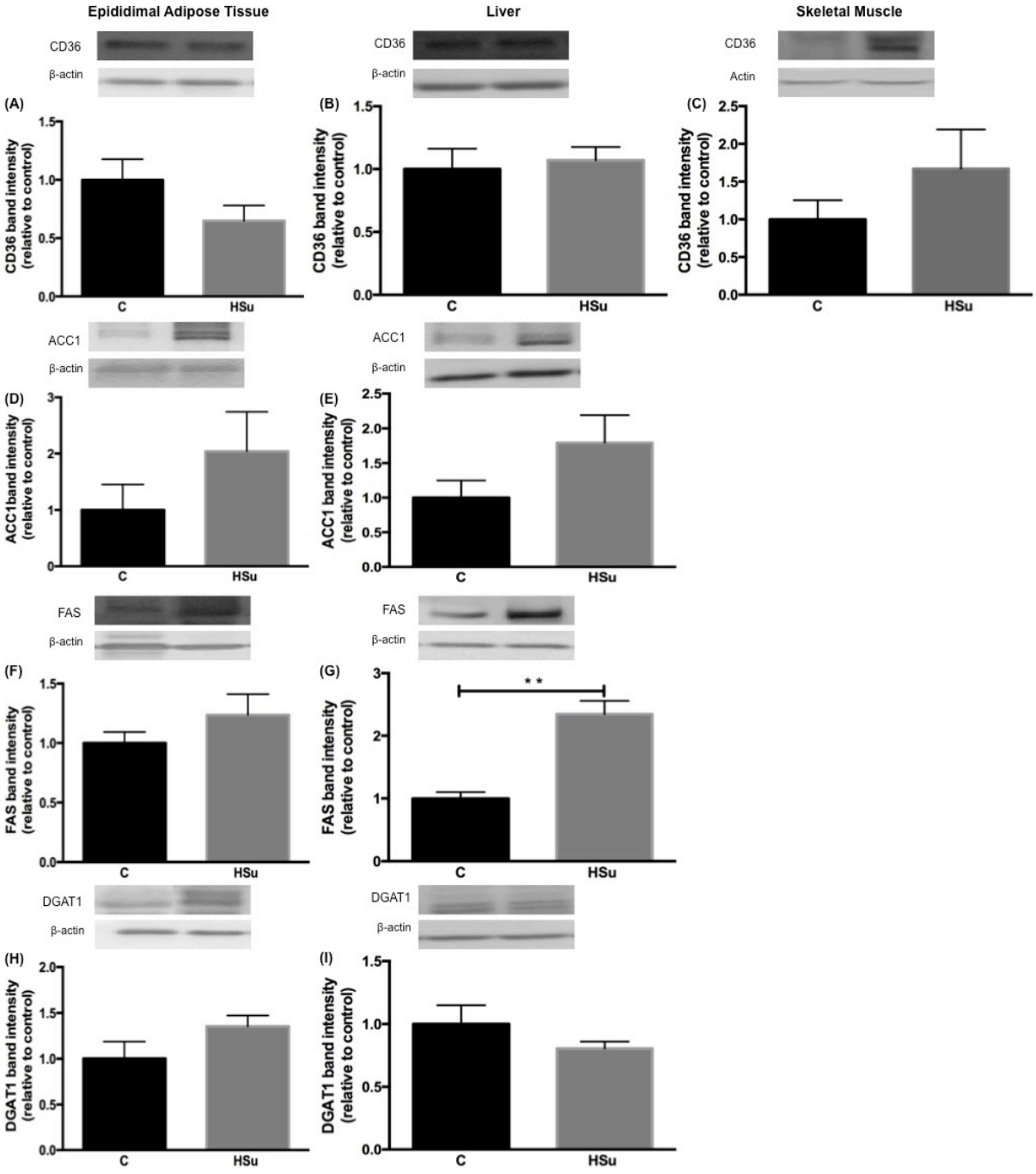

Figure 18. ***Effect of a diet enriched in sucrose (HSu) in proteins.*** CD36 in adipose tissue **(A),** CD36 in liver **(B)**, CD36 in skeletal muscle **(C),** ACC1 in adipose tissue **(D),** ACC1 in liver, **(E)** FAS in adipose tissue **(F),** FAS in liver **(G)**, DGAT1 in adipose tissue **(H)**, DGAT1 in adipose tissue **(I)** levels**.** Data are expressed as mean ± SEM, n=6 for Control groups and n=5 for HSu groups. C, Control. t-test, ** $p \leq 0.01$.

6. Glucose metabolism in the liver

In the liver, were also assessed and PEPCK, which catalyzes the first step in hepatic gluconeogenesis, however its expression level remains unaltered after this treatment (Figure. 19 A). In addition, in order to understand the role of G6Pase in gluconeogenesis, we have analyzed its expression and it was observed a tendency (1.000 ± 0,1419 *vs 1.665* ± 0.2139, p = 0.0519) for enhanced expression in the HSu group when compared to the control group (Figure. 19 B).

Due to GK's role in glycogen synthesis, we studied GK expression by western blot. However, no significant differences were found (Figure. 19 C).

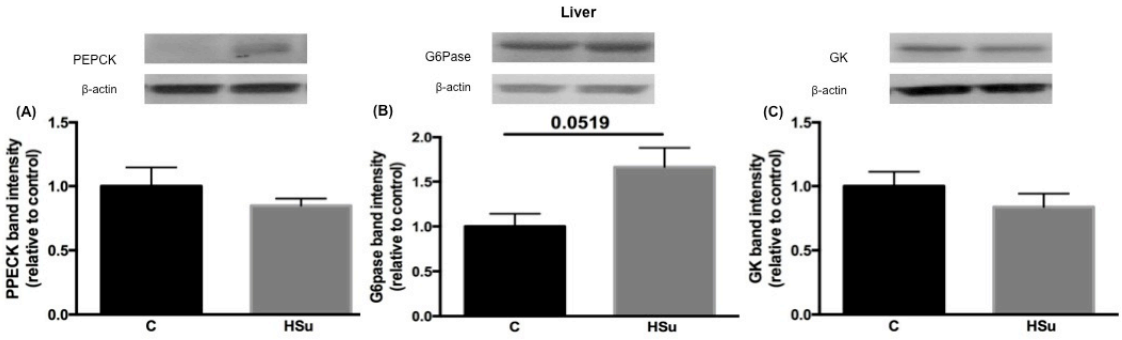

Figure 19. ***Effect of a diet enriched in sucrose (HSu) glucose metabolism in the liver. PEPCK (A), G6Pase (B), and GK (C).*** Data are pressed as mean ± SEM, n=6 for Control groups and n=5 for HSu groups. C, Control. t-test.

IV. Discussion

IV. DISCUSSION

Our study provides two major novel findings: at least in part both gluconeogenesis and lipogenesis are altered in the state of prediabetes/insulin resistance in a model of chronic HSu intake.

As previously described by our group, our animal model presents hypertriglyceridemia, hyperinsulinemia, fasting normoglycemia; however, HSu rats have no alterations in blood pressure neither in total cholesterol levels [94]. In other studies, with male Wistar rats under a high sucrose diet (30%) for 24 weeks also showed no alterations in body weigh and also maintain normoglycemia. However, hypertriglyceridemia, hypertension were higher in high sucrose diet group and a tendency for insulin resistance were observed in this model, which is accords with our findings, however the treatment was longer [95].

In addition, HSu treated animals did not revealed alterations in body weight, showing no signs of obesity; nevertheless, they showed evidence of impaired insulin tolerance during a GTT, which could indicate features of insulin resistance. Furthermore, in isolated adipocytes, insulin-stimulated glucose uptake was decrease in treated animals relative to control animals. Isoproterenol-stimulated lipolysis also pointed to impairment in the antilipolytic insulin effect. FAS and G6Pase were increased in liver of HSu treated animals indicating that both gluconeogenesis and lipogenesis are altered. Moreover, GLUT1 expression in liver was decreased indicating possible alterations in glucose transport activity in liver.

In the present study, we attempt to find metabolic alterations associated with insulin resistance at an early state before the onset of diabetes and perhaps eve more importantly find such alterations in the early stages of insulin resistance, to accomplish this, we used an animal model partially characterized previously by our

group, the HSu-treated rats [94], obtained under a diet enriched in sucrose for a period of 9 weeks.

The Hsu treated animals, male Wistar rats with 16 weeks at the beginning of the study, didn't develop obesity, when compared to the control group, in agreement with previous studies [96], making this an important model to study insulin resistance in a lean phenotype, making it possible to study more in depth the mechanisms involved in insulin resistance and early development of diabetes without confounding factors relative to obesity. Although, other study with, also, male Wistar rats have reported different results; in those studies, rats were subjected to a longer exposure to a high sucrose diet (30% sucrose in drinking water), about 21 weeks increasing their body weight compared to control [97]. However, in other study, where were used Male C57BL/6J mice (4 weeks old), under a high sucrose diet (50% enriched sucrose water) for 55 weeks, animals didn't develop obesity, comparing to control [98].

During the nine week treatment, although drinking more sucrose enriched water, the HSu treated animals have consumed less chow than the control group. This was also reported before by Yi-Chun Chou and colleagues [99], where the consumption of chow was reduced by 57% in the treated group, with 30% sucrose enriched water, leading to higher energy intake, however on a lower nutrient consumption [100]. This is also demonstrated in other studies where it was clear that sucrose contributes to satiety and suppresses subsequent food intake [101]. The lack of nutrients, probably contributes to the devastating consequences of high sucrose diets, like some of the western diets today. In fact, there is a relationship between diets enriched in sucrose and fructose, a product of sucrose metabolism, and the observed insulin resistance [98], [102], as we found in our study with a significant increase in glucose during the

ITT, at both 0 and 120 minutes, as well as a slower glucose excursion during a GTT, with glucose levels significantly increased at 30 and 60 minutes, revealing glucose intolerance. Significantly higher glucose values during a GTT in prediabetic subjects has also been observed in other studies [103], [104] as this alteration is used as a key feature to characterize prediabetes. In addition, several studies performed in lean and healthy relatives of type 2 diabetic subjects have also shown similar results regarding insulin resistance with high fasting glucose and insulin during a oral GTT, in addition to a significantly lower glucose infusion rate during an euglycemic clamp [16], [51].

Furthermore, previous studies with this model [94] showed a hyperinsulinemic state, possibly to compensate the impaired glucose tolerance which, in turn, results from peripheral insulin resistance. This also was observed in similar other models, such as the C57BL/6J mouse, with 7 weeks of age at the beginning of the study, that were submitted to a high sucrose diet (30% sucrose enriched water) for 25 weeks [99]. This hyperinsulinemia is sufficient to counteract the glucose intolerance and keep fasting normal glucose levels. Studies with glucose intolerant and insulin resistant patients with chronic renal failure have also shown that they can enhance their insulin secretion sufficiently to maintain normal glucose tolerance despite glucose intolerance [105]. In other words, insulin resistant subjects can maintain normal glucose levels if the pancreas can keep higher insulin levels that are needed to maintain physiological serum glucose levels. However, when the pancreas begins to fail, insulin secretion and normal serum glucose levels cannot be maintained and patients become diabetic [106]. Thus, this hyperinsulinemia justified itself in this insulin resistant state that we have studied. Other studies have shown that high sucrose diets induce insulin resistance [107] and that metabolic changes are

common in this diet, with hypertriglyceridemia occurring between week one and two and animals becoming hyperinsulinemic and insulin resistant after two weeks of starting the diet [108].

Serum NEFA concentration did not present significant differences between HSu and control rats, in the fed state, however, in the fasted state, in the presence of exogenous insulin NEFA levels were decreased in the HSu treated animals. Other studies have shown that chronic elevation of NEFA is associated with reduced insulin synthesis [29].

Ex vivo studies performed in our laboratory evaluating insulin-stimulated glucose uptake in isolated epididimal adipocytes, we found that both groups of animals responded to the stimulatory effect of insulin on glucose uptake; however, we have found that insulin's effect was different between the control and HSu treated groups. We found an unexpectedly high basal (non-insulin stimulated) glucose uptake in the control group compared to the basal in the HSu treated group. However the insulin-stimulated effect in the control group was much higher than that observed in the Hsu treated group. In view of this, our next objective was to evaluate glucose metabolism in HSu treated animals, namely, the expression of glucose transporters, as well as the expression of important proteins involved in the insulin-mediated signaling pathway involved in glucose uptake and glucose metabolism, in the three main insulin sensitive target tissues, namely adipose tissue, liver and skeletal muscle. In our present study, we observed that GLUT1 was decreased while G6Pase protein content was increased, in the liver. In agreement with our results, previous studies have reported that diabetes causes induction of GLUT1 expression in the plasma membrane of rat hepatocytes and that chronic insulin treatment of diabetic rats reduces the GLUT1 expression [109]. Since our animal model is

characterized by hiperinsulinemia [94], this may reduce the expression levels of GLUT1 in the liver of HSu treated animals. Moreover, in our previous studies we also observed a significant decrease in GLUT1 expression in liver of rats treated with either Cyclosporin A or Sirolimus, rendering rats insulin resistant, in similarity to our HSu model [110].

Regarding GLUT2 (in liver) and GLUT4 (in adipose tissue and skeletal muscle), we observed no alterations in their protein levels in any of the studied tissues. The translocation of GLUT2 and GLUT4 to the membrane is mediated by insulin, which leads to increased glucose uptake. In fact, previous studies reported that insulin can target sugar absorption by controlling the membrane localization of GLUT2 [111]. Similarly, in hyperinsulinemic-euglycemic clamp experiments in responsive to insulin mice, insulin decreased plasma membrane expression of GLUT2, and concomitantly increased intracellular GLUT2 levels [111]. Moreover, acute insulin treatment before sugar intake prevented the translocation of GLUT2 into the plasma membrane [111]. In addiction, insulin resistance in mice provoked a loss of GLUT2 trafficking [111]. Regarding GLUT4, insulin exerts systemic hypoglycaemic effects by stimulating the translocation of GLUT4 into the plasma membrane of skeletal muscle and adipose cells and decreasing liver glucose output [111]. In fact, we evaluated GLUT2 and GLUT4 protein levels rather than its intracellular localization. Thus, in upcoming studies, we will evaluate the internalization of these transporters *versus* its translocation to the plasma membrane in response to insulin stimulation.

In similar studies were the effect of insulin on glucose transport, glucose transporter 4 translocation, and intracellular signaling were measured in fat cells from lean and obese Zucker rats of different ages, it was found that the insulin resistance in fat

cells from old and obese Zucker rats can be accounted for by an impaired GLUT4 translocation process, due to signaling defects leading to a reduced activation of PI3-kinase and PKB, as well as an attenuated GLUT4 protein content in fat [112].

Insulin resistance is characterized by the alteration of the insulin-mediated activation of the PI3K/PKB/Akt signaling pathway [58]. Animal studies links insulin resistance with defects to both upstream and downstream targets of Akt/PKB [113]. Impaired activation of PKB/Akt in response to insulin has been then described in insulin-resistant human, showing that the ability of insulin to increase glucose transport and activate PKB/Akt is reduced in fat cells from T2DM subjects [114] and rodent adipocytes, showing that insulin resistance in cells from old and obese Zucker rats is a result of signaling defects leading to a reduced activation of PI3K and PKB/Akt [112]. However, in our study, we observed no signs of alterations in the phosphorylation/activation of PKB/Akt by insulin, possibly because we needed to increase the number of animals in our study.

Although, in our model, PGC-1α showed no alterations in its protein level, it has been implicated in the onset of T2DM. In liver, where it promotes activation of gluconeogenesis and fatty acid oxidation, its expression is elevated in T2DM mouse models [115]. On the other hand, in humans, reduced adipose PGC-1α content and an association between reduced PGC-1α mRNA levels and insulin resistance were observed [116]. Moreover, in our previous studies PGC-1α was significantly reduced in the three target tissues [110].

FOXO1 also promotes gluconeogenesis, regulating glucose production in the liver. Insulin resistance leads to elevated FOXO1 activation, which upregulates genes involved in glucose production, increasing serum glucose levels [117].

Although, G6Pase, which is involved in gluconeogenesis, was increased, no alterations were observed in FOXO1.

Moreover, in previous studies, it was reported that patients with IFG in fasted state had higher rates of gluconeogenesis [65]. Accordingly, we have observed an increase in G6Pase expression levels in liver from HSu-treated rats. In fact, this enzyme completes the final step in gluconeogenesis and therefore plays a key role in the homeostatic regulation of blood glucose levels. Since HSu-treated rats appear to be a model of insulin resistance, insulin cannot inhibit the *de novo* glucose production in the liver, leading therefore to an increase in gluconeogenesis. As a compensatory mechanism, the pancreas of HSu-treated animals secretes higher levels of insulin to reduce plasma glucose levels, which translates into a previously reported fasting normoglicemia. In the fed state, blood glucose levels raise, leading to an increased insulin secretion by the pancreas. However, since this animal model presents hyperinsulinemia and insulin resistance, the metabolic responses to insulin are altered, resulting in elevated blood glucose in the fed state, as previously reported [109], as well as altered glucose tolerance during a GTT.

In T2DM, glycogen synthesis is also impaired, as the expression of GK is lower than normal, which contributes to hyperglicemia [118], however, in our study GK expression shows no impairment under this condition.

Furthermore, regarding lipid metabolism, in our *ex vivo* studies which evaluated isoproterenol-stimulated lipolysis in isolated epididimal adipocytes, we found that both groups of animals responded to the stimulatory effect of isoproterenol on lipolysis. In fact, we observed a significant increase in isoproterenol-stimulated lipolysis in both control and HSu rats. Moreover, the induction of lipolysis was largely increased in HSu rats compared to that of control animals. Contrary to

what we expected, we found that insulin did not inhibit lipolysis in these animals. Actually, visceral adipocytes appear to be more sensitive to stimulation of lipolysis by catecholamines and less to suppression of lipolysis by insulin. This could lead to an increased free fatty acid flow to the muscle and liver, contributing to an increase in TG content in liver and intramyocellular level, and, at the end, to the insulin resistance previously reported in our HSu model [94] and Zucker fatty (fa/fa) rats that have metabolic abnormalities characteristic for prediabetic condition [119],[94].

In order to explain the lipolysis results, we evaluated the protein levels of the lipolysis-rate limiting enzyme, HSL, as well as proteins involved in lipogenesis. Regarding HSL expression, we have found no differences in protein content of this enzyme between the two groups of animals. Importantly, the protein expression does not correspond to lipolytic activity. Actually, HSL is regulated by reversible phosphorylation on five critical residues [120]. Thus, in our upcoming studies, we will measure HSL's enzymatic activity after isoproterenol stimulation in epididimal adipose tissue.

Moreover, regarding fatty acid uptake, we have studied CD36 and no differences were found in its protein content. Mice lacking CD36 exhibit increased plasma free fatty acid and triglyceride (TG) levels and decreased glucose levels. A deficiency of this protein is associated with an increase of insulin sensitivity in muscle and induction of insulin resistance in mice liver [121]. In previous studies with T2DM patients, CD36 protein was upregulated in fat tissue [122].

In this study we have also evaluated transcription factors involved in glucose and lipid metabolism. ChREBP is regulated by glucose and it modulates the conversion of glucose into fatty acids, reducing plasma glucose levels [123]. In

adipose tissue of HSu animals, we found a significant increase of this protein, which could explain the hypertriglyceridemia associated to this animal model.

Despite previous studies showed SREBP overexpression, a transcription factor that activates fatty acid synthesis, in both liver and adipose tissue of insulin resistance and diabetic mice [124], [125] we did not found any alteration in our study.

PPARs have been implicated in metabolic pathways such as lipid and glucose homeostasis. PPARα activation leads to fatty acid oxidation, improving insulin sensitivity by reducing lipid accumulation in tissues [126]. PPARβ is involved in adipogenesis and, studies in diabetic rats, have found that its activation reduces the production of pro-inflammatory cytokines involved in the development of insulin resistance [127]. PPARγ is expressed in fat and its involved in glucose and lipid uptake, stimulates glucose oxidation, and decreases free fatty acid levels. Synthetic ligands for PPARα and γ, such as thiazolidinediones, have been used in T2DM and prediabetic insulin resistance patients with significantly improved HbA1c and serum glucose levels [128]. In this study we noticed no changes in the protein content of any of the PPAR isoforms studied in adipose tissue.

Regarding lipogenesis, we studied ACC1, an isoform of ACC, that catalyzes the irreversible reaction of fatty acid synthesis by carboxylating acetyl CoA to produce malonyl-CoA [129] and it is known that starvation and diabetes decrease ACC1 activity, and refeeding with a carbohydrate diet induces the synthesis and activity of ACC1 [130], however we didn't find significant alterations in ACC1 between control and treated animals on our study, there was a tendency for an increase in both fat and liver, similar to what we found in fat cells of insulin resistant animals treated with either Cyclosporin A or Sirolimus [110].

In addition, we observed increased FAS expression levels in the liver of HSu-treated animals, in part explaining the hypertriglyceridemia observed in the HSu treated animals [94]. This increase has also been found in other studies where high sucrose diets were used [131], [132]. Sucrose is a disaccharide that is efficiently hydrolyzed by sucrase in the intestinal mucosa to its constituent monosaccharides, fructose and glucose. It has been established that glucose stimulates fructose uptake in a dose-dependent manner [133] and that monosaccharides derived from sucrose are essentially absorbed at a similar rate to glucose:fructose mixtures [134]. Fructose is mainly metabolized in the liver and may be converted into trioses that can be used for *de novo* synthesis of triglycerides (TG) and cholesterol [135], [136]. Fructose, by providing large amounts of hepatic triose-phosphate as precursors for fatty acid synthesis, is highly lipogenic [102]. Therefore, it has been observed in several studies that hepatic *de novo* lipogenesis is stimulated after acute fructose ingestion, with fructose contributing to the synthesis of both the glycerol and the fatty-acyl parts of VLDL-triglycerides [137], [138]. Moreover, fructose may increase the expression of key lipogenic enzymes in the liver. In fact, hypertriglyceridemia has been long known to be associated with insulin resistance in metabolic syndrome [139] and other metabolic diseases, such as T1DM, T2DM and dyslipidemia.

DGAT1 is an isoform of DGAT enzyme known to catalyse the final step of triglyceride synthesis in mammalian [140]. Other studies have shown that DGAT1 deficiency enhances insulin signaling in peripheral tissues and enhances insulin action in white adipose tissue [141]. Despite our animal model present hypertriglyceridemia, we didn't find significant differences on DGAT1 protein levels.

Although more studies are required to clarify the biomolecular mechanisms regulating glucose and lipid metabolism in HSu-treated rats, our study identified

important new alterations in glucose and lipid metabolism that are responsible at least in part for the dysregulated metabolism observed in our prediabetic insulin resistant model.

V. Conclusion

V. CONCLUSION

This study brings new fundamental insights, regarding the development of insulin resistance and prediabetes and its associated comorbidities, such as the metabolic syndrome, [94], [88].

Our animal model treated chronically with a high sucrose diet that mimics at least in part the western diet [94], [142], shows impairments in glucose tolerance, insulin sensivity and in the insulin mediated uptake of glucose into fat. Moreover, gluconeogenic and lipogenic mechanisms revealed, already, similar features of T2DM.

This study is an important wake up call for the lifestyle that general population are gradually adopting, consuming each time more simples sugars, that are contained, for example in soft drinks [143], which has been proven that enhances the risk of develop T2DM to 26% if the average intake is one/two cans a day, or even more [144]. These feeding habits are some of the main causes of the uncontrolled increase in T2DM and associated complication, as coronary heart disease [145] and insulin resistance [146]. Alterations in the western diet need to be taken into consideration to avoid the concerning forecasted numbers [6]. Corrections of this lifestyle features, as decreasing sugar-sweetened beverage consumption, has been proven as effective in decreasing the risk to developing T2DM [143].

VI. References

VI. REFERENCES

[1] Z. Tao, A. Shi, and J. Zhao, “Epidemiological Perspectives of Diabetes,” *Cell Biochem. Biophys.*, Feb. 2015.

[2] W. H. Organisation, “Who: the Top Ten Causes of Death,” no. February, pp. 1–5, 2007.

[3] B. Allgot, D. Gan, H. King, P. Lefèbvre, J.-C. Mbanya, M. Silink, L. Siminerio, R. Williams, and P. Zimmet, *Diabetes Atlas*, 2th edn. 2003.

[4] A. D. Association, *Standards of Medical Care in Diabetes 2015*, vol. 38, no. January. 2015.

[5] J. W. Stevens, K. Khunti, R. Harvey, M. Johnson, L. Preston, H. B. Woods, M. Davies, and E. Goyder, “Preventing the progression to Type 2 diabetes mellitus in adults at high risk: A systematic review and network meta-analysis of lifestyle, pharmacological and surgical interventions,” *Diabetes Res. Clin. Pract.*, vol. 107, no. 3, pp. 320–331, Mar. 2015.

[6] “IDF Diabetes Atlas 2014 update,” 6th edn., 2014.

[7] O. Ozougwu, “The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus,” *J. Physiol. Pathophysiol.*, vol. 4, no. 4, pp. 46–57, Sep. 2013.

[8] Observatório nacional da Diabetes, “Diabetes: factos e números, Relatório anual do observatório nacional da Diabetes 2014,” *Obs. Nac. da Diabetes*, 2014.

[9] Z. Jorge, “Pré diabetes Diagnóstico e Tratamento.”

[10] R. I. G. Holt, C. S. Cockram, A. Flyvbjerg, and B. J. Goldstein, Eds., *Textbook of Diabetes*, 4th edn. Oxford, UK: Wiley-Blackwell, 2010.

[11] IDF Diabetes Atlas, “What is Diabetes,” 6th Edn, Ed. .

[12] M. Ahmed, M. Yaseen, and J. Zafar, “Metabolic Syndrome in Non- Diabetic First Degree Relatives of Type 2 Diabetic Patients,” *Ann. Pakistan Inst. Med. Sci.*, vol. 7, no. 2, pp. 65–71, 2007.

[13] U. Smith, M. Axelsen, E. Carvalho, B. Eliasson, P. a Jansson, and C. Wesslau, “Insulin signaling and action in fat cells: associations with insulin resistance and type 2 diabetes.,” *Ann. N. Y. Acad. Sci.*, vol. 892, pp. 119–126, Nov. 1999.

[14] J. V. Neel, “Diabetes mellitus: a ‘thrifty’ genotype rendered detrimental by ‘progress’? 1962.,” *Bull. World Health Organ.*, vol. 77, no. 8, 1999.

[15] G. Raana and R. Khurshid, “A new look at screening and diagnosis of diabetes mellitus in first degree relatives : Role of HbA1c , insulin resistance , metal ions and heat shock proteins,” *J. Diabetol.*, vol. 1, no. 5, pp. 1–7, 2014.

[16] E. Carvalho, P. a Jansson, M. Axelsen, J. W. Eriksson, X. Huang, L. Groop, C. Rondinone, L. Sjöström, and U. Smith, “Low cellular IRS 1 gene and protein expression predict insulin resistance and NIDDM.,” *FASEB J.*, vol. 13, no. 15, pp. 2173–2178, Dec. 1999.

[17] H. K. Brekke, R. a Lenner, M.-R. Taskinen, J.-E. Månsson, T. Funahashi, Y. Matsuzawa, and P.-A. Jansson, “Lifestyle modification improves risk factors in type 2 diabetes relatives.,” Apr. 2005.

[18] P.-A. JANSSON, “A novel cellular marker of insulin resistance and early atherosclerosis in humans is related to impaired fat cell differentiation and low adiponectin,” *FASEB J.*, vol. 17, no. 11, pp. 1434–1440, Aug. 2003.

[19] M. Straczkowski, I. Kowalska, A. Stepień, S. Dzienis-Straczkowska, M. Szelachowska, I. Kinalska, A. Krukowska, and M. Konicka, “Insulin resistance in the first-degree relatives of persons with type 2 diabetes.,” *Med. Sci. Monit.*, vol. 9, no. 5, pp. CR186–R190, 2003.

[20] X. Yang, P. A. Jansson, I. Nagaev, M. M. Jack, E. Carvalho, K. S. Sunnerhagen, M. C. Cam, S. W. Cushman, and U. Smith, “Evidence of impaired adipogenesis in insulin resistance,” *Biochem. Biophys. Res. Commun.*, vol. 317, no. 4, pp. 1045–1051, May 2004.

[21] H. M. Georgiou, M. Lappas, G. M. Georgiou, A. Marita, V. J. Bryant, R. Hiscock, M. Permezel, Z. Khalil, and G. E. Rice, “Screening for biomarkers predictive of gestational diabetes mellitus,” *Acta Diabetol.*, vol. 45, no. 3, pp. 157–165, 2008.

[22] P. J. Rozance, “Update on neonatal hypoglycemia,” *Curr. Opin. Endocrinol. Diabetes. Obes.*, vol. 21, no. 1, pp. 45–50, 2014.

[23] *Atlas of Diabetes*. Springer Science & Business Media, 2012.

[24] N. W. Cheung, A. Lih, S. M. Lau, K. Park, S. Padmanabhan, and A. McElduff, “Gestational diabetes: a red flag for future Type 2 diabetes in pregnancy? A retrospective analysis,” *Diabet. Med.*, Feb. 2015.

[25] R. E. Pratley and G. Matfin, “Review: Pre-diabetes: clinical relevance and therapeutic approach,” *Br. J. Diabetes Vasc. Dis.*, vol. 7, no. 3, pp. 120–129, May 2007.

[26] W. Yang, T. M. Dall, P. Halder, P. Gallo, S. L. Kowal, P. F. Hogan, and M. Petersen, “Economic costs of diabetes in the U.S. in 2012,” *Diabetes Care*, vol. 36, no. 4, pp. 1033–1046, 2013.

[27] R. a. DeFronzo and D. Tripathy, “Skeletal Muscle Insulin Resistance Is the Primary Defect in Type 2 Diabetes,” *Diabetes Care*, vol. 32, no. suppl_2, pp. S157–S163, Nov. 2009.

[28] K. Choi and Y.-B. Kim, “Molecular Mechanism of Insulin Resistance in Obesity and Type 2 Diabetes,” *Korean J. Intern. Med.*, vol. 25, no. 2, p. 119, 2010.

[29] G. Wilcox, “Insulin and insulin resistance.,” *Clin. Biochem. Rev.*, vol. 26, no. 2, pp. 19–39, 2005.

[30] A. Guilherme, J. V. Virbasius, V. Puri, and M. P. Czech, “Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes,” *Nat. Rev. Mol. Cell Biol.*, vol. 9, no. 5, pp. 367–377, May 2008.

[31] N. Turner, G. J. Cooney, E. W. Kraegen, and C. R. Bruce, “Fatty acid metabolism, energy expenditure and insulin resistance in muscle,” *J. Endocrinol.*, vol. 220, no. 2, pp. T61–T79, Jan. 2014.

[32] I. Moreno-Indias and F. J. Tinahones, “Impaired Adipose Tissue Expandability and Lipogenic Capacities as Ones of the Main Causes of Metabolic Disorders,” *J. Diabetes Res.*, vol. 2015, pp. 1–12, 2015.

[33] V. Lambadiari, K. Triantafyllou, and G. D. Dimitriadis, “Insulin action in muscle and adipose tissue in type 2 diabetes: The significance of blood flow.,” *World J. Diabetes*, vol. 6, no. 4, pp. 626–33, 2015.

[34] K. Ghoshal, “Adiponectin: Probe of the molecular paradigm associating diabetes and obesity,” *World J. Diabetes*, vol. 6, no. 1, p. 151, 2015.

[35] A. S. Lihn, T. Østergard, B. Nyholm, S. B. Pedersen, B. Richelsen, and O. Schmitz, “Adiponectin expression in adipose tissue is reduced in first-degree relatives of type 2 diabetic patients.,” *Am. J. Physiol. Endocrinol. Metab.*, vol. 284, no. 2, pp. E443–E448, 2003.

[36] J. Liu, F. Wang, Y. Cha, Z. Chen, and H. Ding, “Adiponectin Levels in Non-obese First-degree Relatives of Type 2 Diabetes Patients and Non-diabetic Subjects: A 5-Year Follow-up Study,” *J. Int. Med. Res.*, vol. 38, no. 3, pp. 792–802, Jun. 2010.

[37] L. Rui, “Energy Metabolism in the Liver,” in *Comprehensive Physiology*, vol. 4, no. 1, Hoboken, NJ, USA: John Wiley & Sons, Inc., 2014, pp. 177–197.

[38] S. M. Grundy, “Pre-Diabetes, Metabolic Syndrome, and Cardiovascular Risk,” *J. Am. Coll. Cardiol.*, vol. 59, no. 7, pp. 635–643, Feb. 2012.

[39] W. A. Hsueh, L. Orloski, and K. Wyne, “Prediabetes: The Importance of Early Identification and Intervention,” *Postgrad. Med.*, vol. 122, no. 4, pp. 129–143, Jul. 2010.

[40] M. Buysschaert and M. Bergman, “Definition of Prediabetes,” *Medical Clinics of North America*, vol. 95, no. 2. Elsevier Ltd, pp. 289–297, 2011.

[41] J. S. Yudkin and V. M. Montori, “The epidemic of pre-diabetes: the medicine and the politics,” *BMJ*, vol. 349, no. jul15 24, pp. g4485–g4485, Jul. 2014.

[42] D. T. Juarez, K. M. Demaris, R. Goo, C. L. Mnatzaganian, and H. Wong Smith, “Significance of HbA1c and its measurement in the diagnosis of diabetes mellitus: US experience.,” *Diabetes. Metab. Syndr. Obes.*, vol. 7, pp. 487–94, 2014.

[43] D. M. Nathan, B. Balkau, E. Bonora, K. Borch-Johnsen, J. B. Buse, S. Colagiuri, M. B. Davidson, R. DeFronzo, S. Genuth, R. R. Holman, L. Ji, S. Kirkman, W. C. Knowler, D. Schatz, J. Shaw, E. Sobngwi, M. Steffes, O. Vaccaro, N. Wareham, B. Zinman, and R. Kahn, “International Expert Committee Report on the Role of the A1C Assay in the Diagnosis of Diabetes,” *Diabetes Care*, vol. 32, no. 7, pp. 1327–1334, Jul. 2009.

[44] R. A. Martins, J. G. Jones, S. P. Cumming, M. J. Coelho e Silva, A. M. Teixeira, and M. T. Veríssimo, “Glycated hemoglobin and associated risk factors in older adults,” *Cardiovascular Diabetology*, vol. 11, no. 1. p. 13, 2012.

[45] K.-D. Kohnert, “Utility of different glycemic control metrics for optimizing management of diabetes,” *World J. Diabetes*, vol. 6, no. 1, p. 17, 2015.

[46] M. Incani, F. Sentinelli, L. Perra, M. G. Pani, M. Porcu, A. Lenzi, M. G. Cavallo, E. Cossu, F. Leonetti, and M. G. Baroni, “Glycated hemoglobin for the diagnosis of diabetes and prediabetes: Diagnostic impact on obese and lean subjects, and phenotypic characterization,” *J. Diabetes Investig.*, vol. 6, no. 1, pp. 44–50, Jan. 2015.

[47] A. G. Tabák, C. Herder, W. Rathmann, E. J. Brunner, and M. Kivimäki, “Prediabetes: A high-risk state for diabetes development,” *Lancet*, vol. 379, no. 9833, pp. 2279–2290, Jul. 2012.

[48] E. Ferrannini, “Definition of intervention points in prediabetes,” *The Lancet Diabetes and Endocrinology*, vol. 8587, no. 13, pp. 1–9, 2014.

[49] K. C. Portero McLellan, K. Wyne, E. T. Villagomez, and W. A. Hsueh, “Therapeutic interventions to reduce the risk of progression from prediabetes to type 2 diabetes mellitus,” *Therapeutics and Clinical Risk Management*, vol. 10, no. 1. pp. 173–188, Mar-2014.

[50] S. Colagiuri, “Epidemiology of Prediabetes,” *Medical Clinics of North America*, vol. 95, no. 2. pp. 299–307, 2011.

[51] E. Carvalho, P. A. Jansson, I. Nagaev, A. M. Wenthzel, and U. Smith, “Insulin resistance with low cellular IRS-1 expression is also associated with low GLUT4 expression and impaired insulin-stimulated glucose transport.,” *FASEB J.*, vol. 15, no. 6, pp. 1101–1103, Feb. 2001.

[52] H. Abe, N. Yamada, K. Kamata, T. Kuwaki, M. Shimada, J. Osuga, F. Shionoiri, N. Yahagi, T. Kadowaki, H. Tamemoto, S. Ishibashi, Y. Yazaki, and M. Makuuchi, “Hypertension, hypertriglyceridemia, and impaired endothelium-dependent vascular relaxation in mice lacking insulin receptor substrate-1.,” *J. Clin. Invest.*, vol. 101, no. 8, pp. 1784–1788, Apr. 1998.

[53] L. Plum, “Transgenic and Knockout Mice in Diabetes Research: Novel Insights into Pathophysiology, Limitations, and Perspectives,” *Physiology*, vol. 20, no. 3, pp. 152–161, Jun. 2005.

[54] S. I. T. M. P. erek LeRoith MD PhD, Jerrold M. Olefsky MD, *Diabetes Mellitus: A Fundamental and Clinical Text*, 3rd edn. Lippincott Williams & Wilkins, 2003.

[55] C. M. Taniguchi, B. Emanuelli, and C. R. Kahn, “Critical nodes in signalling pathways: insights into insulin action,” *Nat. Rev. Mol. Cell Biol.*, vol. 7, no. 2, pp. 85–96, Feb. 2006.

[56] J. M. Lizcano and D. R. Alessi, “The insulin signalling pathway,” *Curr. Biol.*, vol. 12, no. 7, pp. R236–R238, Apr. 2002.

[57] H. K. R. Karlsson, J. R. Zierath, S. Kane, A. Krook, G. E. Lienhard, and H. Wallberg-Henriksson, “Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects,” *Diabetes*, vol. 54, no. 6, pp. 1692–1697, 2005.

[58] S. Fröjdö, H. Vidal, and L. Pirola, “Alterations of insulin signaling in type 2 diabetes: A review of the current evidence from humans,” *Biochim. Biophys. Acta - Mol. Basis Dis.*, vol. 1792, no. 2, pp. 83–92, Feb. 2009.

[59] J. P. McClung, C. a Roneker, W. Mu, D. J. Lisk, P. Langlais, F. Liu, and X. G. Lei, “Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 24, pp. 8852–8857, Jun. 2004.

[60] L. Chang and S.-H. Chiang, “Insulin Signaling and the Regulation of Glucose Transport,” *Mol. Med.*, vol. 12, no. 7–12, p. 1, 2006.

[61] H. Y. Wang, S. Ducommun, C. Quan, B. Xie, M. Li, D. Wasserman, K. Sakamoto, C. MacKintosh, and S. Chen, “AS160 deficiency causes whole-body insulin resistance via composite effects in multiple tissues,” *Biochemical Journal*, vol. 449, no. 2. pp. 479–489, 15-Jan-2012.

[62] D. L. Nelson and Michael M. Cox, *Lehninger Principles of Biochemistry*, Sixth Edit. Freeman, W. H. & Company, 2012.

[63] C. Bouché, S. Serdy, C. R. Kahn, and A. B. Goldfine, “The Cellular Fate of Glucose and Its Relevance in Type 2 Diabetes,” *Endocr. Rev.*, vol. 25, no. 5, pp. 807–830, Oct. 2004.

[64] I. Toft and T. Jenssen, “Type 2 diabetic patients have increased gluconeogenic efficiency to substrate availability, but intact autoregulation of endogenous glucose production.,” *Scand. J. Clin. Lab. Invest.*, vol. 65, no. 9038, pp. 307–320, 2005.

[65] R. Basu, C. Barosa, J. Jones, S. Dube, R. Carter, A. Basu, and R. a. Rizza, “Pathogenesis of Prediabetes: Role of the Liver in Isolated Fasting Hyperglycemia and Combined Fasting and Postprandial Hyperglycemia,” *J. Clin. Endocrinol. Metab.*, vol. 98, no. 3, pp. E409–E417, Mar. 2013.

[66] M.-R. Taskinen and J. Borén, “New insights into the pathophysiology of dyslipidemia in type 2 diabetes,” *Atherosclerosis*, vol. 239, no. 2, pp. 483–495, Apr. 2015.

[67] S. Kersten, “Mechanisms of nutritional and hormonal regulation of lipogenesis,” *EMBO Rep.*, vol. 2, no. 4, pp. 282–286, Apr. 2001.

[68] B. C. HANSEN and G. BRAY A., *The metabolic syndrome epidemiology, clinical treatment, and underlying mechanisms*. Humana Press; 2008 edition (January 18, 2008), 2008.

[69] S. Summermatter, O. Baum, G. Santos, H. Hoppeler, and C. Handschin, “Peroxisome Proliferator-activated Receptor Coactivator 1 (PGC-1) Promotes Skeletal Muscle Lipid Refueling in Vivo by Activating de Novo Lipogenesis and the Pentose Phosphate Pathway,” *J. Biol. Chem.*, vol. 285, no. 43, pp. 32793–32800, Oct. 2010.

[70] S. Kleiner, R. J. Mepani, D. Laznik, L. Ye, M. J. Jurczak, F. R. Jornayvaz, J. L. Estall, D. Chatterjee Bhowmick, G. I. Shulman, and B. M. Spiegelman, “Development of insulin resistance in mice lacking PGC-1 in adipose tissues,” *Proc. Natl. Acad. Sci.*, vol. 109, no. 24, pp. 9635–9640, Jun. 2012.

[71] T. a. Pietka, T. Schappe, C. Conte, E. Fabbrini, B. W. Patterson, S. Klein, N. a. Abumrad, and L. Love-Gregory, “Adipose and Muscle Tissue Profile of CD36 Transcripts in Obese Subjects Highlights the Role of CD36 in Fatty Acid Homeostasis and Insulin Resistance,” *Diabetes Care*, vol. 37, no. 7, pp. 1990–1997, Jul. 2014.

[72] P. Steneberg, A. G. Sykaras, F. Backlund, J. Straseviciene, I. Soderstrom, and H. Edlund, “Hyperinsulinemia enhances hepatic expression of the fatty acid transporter Cd36 and provokes hepatosteatosis and hepatic insulin resistance,” *J. Biol. Chem.*, p. jbc.M115.640292, Jun. 2015.

[73] C. Dong, H. Zhou, C. Shen, L. Yu, Y. Ding, Y. Zhang, and Z. Guo, “Role of peroxisome proliferator-activated receptors gene polymorphisms in type 2 diabetes and metabolic syndrome.,” *World J. Diabetes*, vol. 6, no. 4, pp. 654–61, 2015.

[74] C. J. Villanueva, M. Monetti, M. Shih, P. Zhou, S. M. Watkins, S. Bhanot, and R. V. Farese, “Specific role for acyl CoA:Diacylglycerol acyltransferase 1

(Dgat1) in hepatic steatosis due to exogenous fatty acids," *Hepatology*, vol. 50, no. 2, pp. 434–442, Aug. 2009.

[75] L. Reshef, Y. Olswang, H. Cassuto, B. Blum, C. M. Croniger, S. C. Kalhan, S. M. Tilghman, and R. W. Hanson, "Glyceroneogenesis and the Triglyceride/Fatty Acid Cycle," *J. Biol. Chem.*, vol. 278, no. 33, pp. 30413–30416, Aug. 2003.

[76] U. D. of H. and H. Services and Cdc, *Health, United States, 2008, special feature on health of young adults*. 2009.

[77] H. Needs, "History of the Dietary Guidelines for Americans," 2010.

[78] S. K. Arora and S. I. McFarlane, "No Title," *Nutr. Metab. (Lond).*, vol. 2, no. 1, p. 16, 2005.

[79] A. Esmaillzadeh, M. Kimiagar, Y. Mehrabi, L. Azadbakht, F. B. Hu, and W. C. Willett, "Dietary patterns, insulin resistance, and prevalence of the metabolic syndrome in women.," *Am. J. Clin. Nutr.*, vol. 85, no. 3, pp. 910–918, 2007.

[80] F. B. Hu, S. Liu, and R. M. van Dam, "Diet and risk of Type II diabetes: the role of types of fat and carbohydrate," *Diabetologia*, vol. 44, no. 7, pp. 805–817, Jul. 2001.

[81] S. Rowe, N. Alexander, N. Almeida, R. Black, R. Burns, L. Bush, P. Crawford, N. Keim, P. Kris-Etherton, and C. Weaver, "Food Science Challenge: Translating the Dietary Guidelines for Americans to Bring About Real Behavior Change," *J. Food Sci.*, vol. 76, no. 1, pp. R29–R37, Jan. 2011.

[82] F. F. Samaha, N. Iqbal, P. Seshadri, K. L. Chicano, D. a Daily, J. McGrory, T. Williams, M. Williams, E. J. Gracely, and L. Stern, "A Low-Carbohydrate as Compared with a Low-Fat Diet in Severe Obesity," *N. Engl. J. Med.*, vol. 348, no. 21, pp. 2074–2081, May 2003.

[83] P. a. Dyson, S. Beatty, and D. R. Matthews, "A low-carbohydrate diet is more effective in reducing body weight than healthy eating in both diabetic and non-diabetic subjects," *Diabet. Med.*, vol. 24, no. 12, pp. 1430–1435, Dec. 2007.

[84] J. S. Volek, S. D. Phinney, C. E. Forsythe, E. E. Quann, R. J. Wood, M. J. Puglisi, W. J. Kraemer, D. M. Bibus, M. L. Fernandez, and R. D. Feinman, "Carbohydrate Restriction has a More Favorable Impact on the Metabolic Syndrome than a Low Fat Diet," *Lipids*, vol. 44, no. 4, pp. 297–309, Apr. 2009.

[85] S. B. Sondike, N. Copperman, and M. S. Jacobson, "Effects of a low-carbohydrate diet on weight loss and cardiovascular risk factor in overweight adolescents," *J. Pediatr.*, vol. 142, no. 3, pp. 253–258, Mar. 2003.

[86] N. F. Krebs, D. Gao, J. Gralla, J. S. Collins, and S. L. Johnson, "Efficacy and safety of a high protein, low carbohydrate diet for weight loss in severely obese adolescents," *J. Pediatr.*, vol. 157, no. 2, pp. 252–258, Aug. 2010.

[87] W. S. Yancy, M. K. Olsen, J. R. Guyton, R. P. Bakst, and E. C. Westman, “A low-carbohydrate, ketogenic diet versus a low-fat diet to treat obesity and hyperlipidemia,” *ACC Curr. J. Rev.*, vol. 13, no. 8, pp. 18–19, Aug. 2004.

[88] S. Merat, F. Casanada, M. Sutphin, W. Palinski, and P. D. Reaven, “Western-Type Diets Induce Insulin Resistance and Hyperinsulinemia in LDL Receptor-Deficient Mice But Do Not Increase Aortic Atherosclerosis Compared With Normoinsulinemic Mice in Which Similar Plasma Cholesterol Levels Are Achieved by a Fructose-Rich Diet,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 19, no. 5, pp. 1223–1230, May 1999.

[89] J. Montonen, “Dietary Patterns and the Incidence of Type 2 Diabetes,” *Am. J. Epidemiol.*, vol. 161, no. 3, pp. 219–227, Feb. 2005.

[90] G. Viscogliosi, E. Cipriani, M. L. Liguori, B. Marigliano, M. Saliola, E. Ettorre, and P. Andreozzi, “Mediterranean Dietary Pattern Adherence: Associations with Prediabetes, Metabolic Syndrome, and Related Microinflammation,” *Metab. Syndr. Relat. Disord.*, vol. 11, no. 3, pp. 210–216, Jun. 2013.

[91] E. Carvalho, K. Kotani, O. D. Peroni, and B. B. Kahn, “Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle,” vol. 02215, pp. 551–561, 2005.

[92] D. B. Allison, F. Paultre, C. Maggio, N. Mezzitis, and F. X. Pi-Sunyer, “The use of areas under curves in diabetes research,” *Diabetes Care*, vol. 18, no. 2, pp. 245–250, 1995.

[93] T. Mahmood and P. C. Yang, “Western blot: Technique, theory, and trouble shooting,” *N. Am. J. Med. Sci.*, vol. 4, pp. 429–434, 2012.

[94] S. Nunes, E. Soares, J. Fernandes, S. Viana, E. Carvalho, F. C. Pereira, and F. Reis, “Early cardiac changes in a rat model of prediabetes: brain natriuretic peptide overexpression seems to be the best marker,” *Cardiovasc. Diabetol.*, vol. 12, no. 1, p. 44, Jan. 2013.

[95] A. K. Castellanos Jankiewicz, S. M. Rodríguez Peredo, G. Cardoso Saldaña, E. Díaz Díaz, M. E. Tejero Barrera, L. Del Bosque Plata, and R. Carbó Zabala, “ADIPOSE TISSUE REDISTRIBUTION CAUSED BY AN EARLY CONSUMPTION OF A HIGH SUCROSE DIET IN A RAT MODEL.,” *Nutr. Hosp.*, vol. 31, no. n06, pp. 2546–2553, 2015.

[96] M. Kanazawa, C. Y. Xue, H. Kageyama, E. Suzuki, R. Ito, Y. Namba, T. Osaka, S. Kimura, and S. Inoue, “Effects of a high-sucrose diet on body weight, plasma triglycerides, and stress tolerance.,” *Nutr. Rev.*, vol. 61, no. 5 Pt 2, pp. S27–S33, 2003.

[97] A. A. Aguilera, G. H. Díaz, M. L. Barcelata, O. A. Guerrero, and R. M. O. Ros, “Effects of fish oil on hypertension, plasma lipids, and tumor necrosis factor-α in rats with sucrose-induced metabolic syndrome,” *J. Nutr. Biochem.*, vol. 15, no. 6, pp. 350–357, Jun. 2004.

[98] M. Sumiyoshi, M. Sakanaka, and Y. Kimura, “Chronic intake of high-fat and high-sucrose diets differentially affects glucose intolerance in mice.,” *J. Nutr.*, vol. 136, no. 3, pp. 582–587, 2006.

[99] Y. C. Chou, S. Y. Wang, G. C. Chen, Y. S. Lin, and P. M. Chao, “The functional assessment of alpinia pricei on metabolic syndrome induced by sucrose-containing drinking water in mice,” *Phyther. Res.*, vol. 23, no. 4, pp. 558–563, 2009.

[100] Y. Ritze, G. Bárdos, J. G. D’Haese, B. Ernst, M. Thurnheer, B. Schultes, and S. C. Bischoff, “Effect of High Sugar Intake on Glucose Transporter and Weight Regulating Hormones in Mice and Humans,” *PLoS One*, vol. 9, no. 7, p. e101702, Jul. 2014.

[101] G. H. Anderson and D. Woodend, “Consumption of sugars and the regulation of short-term satiety and food intake.,” *The American journal of clinical nutrition*, vol. 78, no. 4. 2003.

[102] L. Tappy and K.-A. Lê, “Metabolic effects of fructose and the worldwide increase in obesity.,” *Physiol. Rev.*, vol. 90, no. 1, pp. 23–46, 2010.

[103] G. D. V. Giorelli, L. N. De Matos, A. Saado, V. L. Soibelman, and C. B. Dias, “No association between 25-hydroxyvitamin D levels and prediabetes in Brazilian patients. A cross-sectional study,” *Sao Paulo Med. J.*, vol. 133, no. 2, pp. 73–77, Apr. 2015.

[104] N. T. JENKINS and J. M. HAGBERG, “Aerobic Training Effects on Glucose Tolerance in Prediabetic and Normoglycemic Humans,” *Med. Sci. Sport. Exerc.*, vol. 43, no. 12, pp. 2231–2240, Dec. 2011.

[105] F. Fiorini, M. Raffa, E. Patrone, and A. Castelluccio, “Glucose metabolism and chronic renal insufficiency,” *Arch. Ital. Urol. Androl.*, vol. 66, no. 1, pp. 51–56, 1994.

[106] S. D. Mendelson, “Metabolic syndrome and psychiatric illness,” in *Metabolic Syndrome and Psychiatric Illness*, Elsevier, 2008, pp. 49–72.

[107] M. J. Pagliassotti, P. A. Prach, T. A. Koppenhafer, and D. A. Pan, “Changes in insulin action, triglycerides, and lipid composition during sucrose feeding in rats.,” *Am. J. Physiol.*, vol. 271, no. 5 Pt 2, pp. R1319–R1326, 1996.

[108] K. Dutta, D. A. Podolin, M. B. Davidson, and A. J. Davidoff, “Cardiomyocyte dysfunction in sucrose-fed rats is associated with insulin resistance,” *Diabetes*, vol. 50, no. 5, pp. 1186–1192, 2001.

[109] M. Tal, B. B. Kahn, and H. F. Lodish, “Expression of the low Km GLUT-1 glucose transporter is turned on in perivenous hepatocytes of insulin-deficient diabetic rats,” *Endocrinology*, vol. 129, no. 4, pp. 1933–1941, 1991.

[110] a Fuhrmann, P. Lopes, J. Sereno, J. Pedro, D. O. Espinoza, M. J. Pereira, F. Reis, J. W. Eriksson, and E. Carvalho, “Molecular mechanisms underlying the effects of cyclosporin A and sirolimus on glucose and lipid metabolism in liver, skeletal muscle and adipose tissue in an in vivo rat model,” *Biochem. Pharmacol.*, vol. 88, no. 2, pp. 216–228, Mar. 2014.

[111] V. Tobin, M. Le Gall, X. Fioramonti, E. Stolarczyk, A. G. Blazquez, C. Klein, M. Prlgent, P. Serradas, M. H. Cuif, C. Magnan, A. Leturque, and E. Brot-Laroche, “Insulin internalizes GLUT2 in the enterocytes of healthy but not insulin-resistant mice,” *Diabetes*, vol. 57, no. 3, pp. 555–562, 2008.

[112] E. Carvalho, C. Rondinone, and U. Smith, “Insulin resistance in fat cells from obese Zucker rats--evidence for an impaired activation and translocation of protein kinase B and glucose transporter 4.,” *Mol. Cell. Biochem.*, vol. 206, no. 1–2, pp. 7–16, 2000.

[113] Y. Zick, “Insulin resistance: A phosphorylation-based uncoupling of insulin signaling,” *Trends in Cell Biology*, vol. 11, no. 11. pp. 437–441, 2001.

[114] C. M. Rondinone, E. Carvalho, C. Wesslau, and U. P. Smith, “Impaired glucose transport and protein kinase B activation by insulin, but not okadaic acid, in adipocytes from subjects with Type II diabetes mellitus,” *Diabetologia*, vol. 42, no. 7, pp. 819–825, 1999.

[115] S.-H. Koo, H. Satoh, S. Herzig, C.-H. Lee, S. Hedrick, R. Kulkarni, R. M. Evans, J. Olefsky, and M. Montminy, “PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3.,” *Nat. Med.*, vol. 10, no. 5, pp. 530–534, 2004.

[116] S. Kleiner, R. J. Mepani, D. Laznik, L. Ye, M. J. Jurczak, F. R. Jornayvaz, J. L. Estall, D. Chatterjee Bhowmick, G. I. Shulman, and B. M. Spiegelman, “Development of insulin resistance in mice lacking PGC-1 in adipose tissues,” *Proc. Natl. Acad. Sci.*, vol. 109, no. 24, pp. 9635–9640, Jun. 2012.

[117] B. Ponugoti, G. Dong, and D. T. Graves, “Role of forkhead transcription factors in diabetes-induced oxidative stress,” *Experimental Diabetes Research*, vol. 2012. 2012.

[118] R. a. Haeusler, S. Camastra, B. Astiarraga, M. Nannipieri, M. Anselmino, and E. Ferrannini, “Decreased expression of hepatic glucokinase in type 2 diabetes,” *Mol. Metab.*, vol. 4, no. 3, pp. 222–226, Mar. 2015.

[119] S. Lupachyk, P. Watcho, N. Hasanova, U. Julius, and I. G. Obrosova, “Triglyceride, nonesterified fatty acids, and prediabetic neuropathy: role for oxidative–nitrosative stress,” *Free Radic. Biol. Med.*, vol. 52, no. 8, pp. 1255–1263, Apr. 2012.

[120] A. D. Lampidonis, E. Rogdakis, G. E. Voutsinas, and D. J. Stravopodis, “The resurgence of Hormone-Sensitive Lipase (HSL) in mammalian lipolysis,” *Gene*, vol. 477, no. 1–2, pp. 1–11, May 2011.

[121] J. R. Goudriaan, “CD36 deficiency increases insulin sensitivity in muscle, but induces insulin resistance in the liver in mice,” *J. Lipid Res.*, vol. 44, no. 12, pp. 2270–2277, Sep. 2003.

[122] a Bonen, N. N. Tandon, J. F. C. Glatz, J. J. F. P. Luiken, and G. J. F. Heigenhauser, “The fatty acid transporter FAT/CD36 is upregulated in subcutaneous and visceral adipose tissues in human obesity and type 2 diabetes,” *Int. J. Obes.*, vol. 30, no. 6, pp. 877–883, Jun. 2006.

[123] M. A. Herman, O. D. Peroni, J. Villoria, M. R. Schön, N. A. Abumrad, M. Blüher, S. Klein, and B. B. Kahn, “A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism,” *Nature*, vol. 484, no. 7394, pp. 333–338, Apr. 2012.

[124] S.-S. Im, S.-Y. Kang, S.-Y. Kim, H. -i. Kim, J.-W. Kim, K.-S. Kim, and Y.-H. Ahn, “Glucose-Stimulated Upregulation of GLUT2 Gene Is Mediated by Sterol Response Element-Binding Protein-1c in the Hepatocytes,” *Diabetes*, vol. 54, no. 6, pp. 1684–1691, Jun. 2005.

[125] P.-H. Ducluzeau, N. Perretti, M. Laville, F. Andreelli, N. Vega, J.-P. Riou, and H. Vidal, “Regulation by Insulin of Gene Expression in Human Skeletal Muscle and Adipose Tissue: Evidence for Specific Defects in Type 2 Diabetes,” *Diabetes*, vol. 50, no. 5, pp. 1134–1142, May 2001.

[126] P. Ferre, “The Biology of Peroxisome Proliferator-Activated Receptors: Relationship With Lipid Metabolism and Insulin Sensitivity,” *Diabetes*, vol. 53, no. Supplement 1, pp. S43–S50, Feb. 2004.

[127] R. Rodríguez-Calvo, L. Serrano, T. Coll, N. Moullan, R. M. Sánchez, M. Merlos, X. Palomer, J. C. Laguna, L. Michalik, W. Wahli, and M. Vázquez-Carrera, “Activation of peroxisome proliferator-activated receptor β/δ inhibits lipopolysaccharide-induced cytokine production in adipocytes by lowering nuclear factor-κB activity via extracellular signal-related kinase 1/2,” *Diabetes*, vol. 57, no. 8, pp. 2149–2157, 2008.

[128] M. A. Jay and J. Ren, “Peroxisome proliferator-activated receptor (PPAR) in metabolic syndrome and type 2 diabetes mellitus.,” *Curr. Diabetes Rev.*, vol. 3, no. 1, pp. 33–39, 2007.

[129] N. B. Ruderman, A. K. Saha, and E. W. Kraegen, “Minireview: Malonyl CoA, AMP-Activated Protein Kinase, and Adiposity,” *Endocrinology*, vol. 144, no. 12, pp. 5166–5171, Dec. 2003.

[130] M.-H. Ryu and Y.-S. Cha, “The effects of a high-fat or high-sucrose diet on serum lipid profiles, hepatic acyl-CoA synthetase, carnitine palmitoyltransferase-I, and the acetyl-CoA carboxylase mRNA levels in rats.,” *J. Biochem. Mol. Biol.*, vol. 36, no. 3, pp. 312–318, 2003.

[131] K. R. Bruckdorfer, I. H. Khan, and J. Yudkin, “Fatty acid synthetase activity in the liver and adipose tissue of rats fed with various carbohydrates.,” *Biochem. J.*, vol. 129, no. 2, pp. 439–446, 1972.

[132] N. Agheli, M. Kabir, S. Berni-Canani, E. Petitjean, A. Boussairi, J. Luo, F. Bornet, G. Slama, and S. W. Rizkalla, “Plasma lipids and fatty acid synthase activity are regulated by short-chain fructo-oligosaccharides in sucrose-fed insulin-resistant rats.,” *J. Nutr.*, vol. 128, no. 8, pp. 1283–1288, 1998.

[133] J. J. Rumessen and E. Gudmand-Høyer, “Absorption capacity of fructose in healthy adults. Comparison with sucrose and its constituent monosaccharides.,” *Gut*, vol. 27, no. 10, pp. 1161–1168, 1986.

[134] L. Tappy, K. a Lê, C. Tran, and N. Paquot, “Fructose and metabolic diseases: New findings, new questions,” *Nutrition*, vol. 26, no. 11–12, pp. 1044–1049, Nov. 2010.

[135] S. K. Fried and S. P. Rao, “Sugars, hypertriglyceridemia, and cardiovascular disease.,” *The American journal of clinical nutrition*, vol. 78, no. 4. 2003.

[136] K. N. Frayn, S. M. Kingman, W. M. Sherman, S. Khan, Wolever, R. B. McDonald, and M. Dreher, “Dietary sugars and lipid metabolism in humans,” in *American Journal of Clinical Nutrition*, 1995, vol. 62, no. 1 SUPPL.

[137] M. F.-F. Chong, B. A. Fielding, and K. N. Frayn, “Mechanisms for the acute effect of fructose on postprandial lipemia.,” *Am. J. Clin. Nutr.*, vol. 85, no. 6, pp. 1511–1520, 2007.

[138] E. J. Parks, L. E. Skokan, M. T. Timlin, and C. S. Dingfelder, “Dietary sugars stimulate fatty acid synthesis in adults.,” *J. Nutr.*, vol. 138, no. 6, pp. 1039–1046, 2008.

[139] M. E. Daly, C. Vale, M. Walker, K. G. Alberti, and J. C. Mathers, “Dietary carbohydrates and insulin sensitivity: a review of the evidence and clinical implications.,” *Am. J. Clin. Nutr.*, vol. 66, no. 5, pp. 1072–1085, 1997.

[140] H. C. Chen, S. J. Stone, P. Zhou, K. K. Buhman, and R. V Farese, “Dissociation of Obesity and Impaired Glucose Disposal in Mice Overexpressing Acyl Coenzyme A:Diacylglycerol Acyltransferase 1 in White Adipose Tissue,” *Diabetes*, vol. 51, no. 11, pp. 3189–3195, Nov. 2002.

[141] H. C. Chen, M. Rao, M. P. Sajan, M. Standaert, Y. Kanoh, A. Miura, R. V. Farese, and R. V. Farese, “Role of Adipocyte-Derived Factors in Enhancing Insulin Signaling in Skeletal Muscle and White Adipose Tissue of Mice Lacking Acyl CoA:Diacylglycerol Acyltransferase 1,” *Diabetes*, vol. 53, no. 6, pp. 1445–1451, Jun. 2004.

[142] A. Bouchard-Mercier, I. Rudkowska, S. Lemieux, P. Couture, and M.-C. Vohl, “The metabolic signature associated with the Western dietary pattern: a cross-sectional study,” *Nutr. J.*, vol. 12, no. 1, p. 158, 2013.

[143] F. B. Hu, “Resolved: there is sufficient scientific evidence that decreasing sugar-sweetened beverage consumption will reduce the prevalence of obesity and obesity-related diseases,” *Obes. Rev.*, vol. 14, no. 8, pp. 606–619, Aug. 2013.

[144] V. S. Malik, B. M. Popkin, G. A. Bray, J.-P. Despres, W. C. Willett, and F. B. Hu, “Sugar-Sweetened Beverages and Risk of Metabolic Syndrome and Type 2 Diabetes: A meta-analysis,” *Diabetes Care*, vol. 33, no. 11, pp. 2477–2483, Nov. 2010.

[145] T. T. Fung, V. Malik, K. M. Rexrode, J. E. Manson, W. C. Willett, and F. B. Hu, “Sweetened beverage consumption and risk of coronary heart disease in women,” *Am. J. Clin. Nutr.*, vol. 89, no. 4, pp. 1037–1042, Apr. 2009.

[146] A. Lana, F. Rodriguez-Artalejo, and E. Lopez-Garcia, “Consumption of Sugar-Sweetened Beverages Is Positively Related to Insulin Resistance and Higher Plasma Leptin Concentrations in Men and Nonoverweight Women,” *J. Nutr.*, vol. 144, no. 7, pp. 1099–1105, Jul. 2014.