UNSUSPECTED PLAYERS ON THE PATHOPHYSIOLOGY OF PREDIABETES AND NON-ALCOHOLIC FATTY LIVER DISEASE

MARIA JOÃO CARVALHO MENESES OLIVEIRA

Tese para obtenção do grau de Doutor em Mecanismos de Doença e Medicina Regenerativa

Doutoramento em associação entre:

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Maria João Carvalho Meneses Oliveira

Orientadores: **Maria Paula Borges de Lemos Macedo, Professora Associada com Agregação em Ciências da Vida da NOVA Medical School da Universidade NOVA de Lisboa**

Marco Aurélio Gouveia Alves, Investigador Principal do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Inês de Sousa Lima, Investigadora Pós-Doutorada da NOVA Medical School da Universidade NOVA de Lisboa

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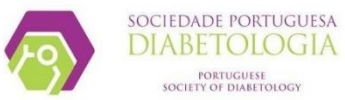

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* the authors contributed equally to the work

To my parents and Magui

"*Health has its science, as well as disease*",

Elizabeth Blackwell

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"Great things are never done by one person, they are done by a team of people",

Steve Jobs

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RESUMO

Nas últimas décadas a prevalência mundial de doenças metabólicas como a obesidade e a diabetes *mellitus* tipo 2 (T2DM) tem aumentado exponencialmente, sendo atualmente uma séria preocupação de saúde, social e económica. Este aumento é paralelo ao aumento do sedentarismo e dos maus hábitos alimentares. De facto, a industrialização e a urbanização levaram ao aumento do consumo de alimentos com alto teor de hidratos de carbono e lípidos. A dieta, juntamente com a predisposição genética, são os principais causadores de desregulação metabólica, que acaba por levar ao aparecimento de complicações metabólicas em vários órgãos, como é o caso do fígado, músculo, tecido adiposo e testículo.

O fígado tem um papel fundamental para a regulação da homeostase da glicose. No estado pós-prandial, o fígado converte a glicose circulante em glicogénio. Por outro lado, no estado de jejum, a glicose é produzida a partir do glicogénio armazenado para manter os níveis de glicose em circulação dentro dos limites normais. De facto, a homeostase sistémica da glicose e da insulina é fortemente regulada por uma rede neuronal e hormonal. A insulina tem um papel central no controlo do metabolismo hepático, estimulando a glicólise e a lipogénese, enquanto suprime a produção endógena de glicose e de corpos cetónicos. Qualquer desequilíbrio no metabolismo energético do fígado pode levar a desregulações no metabolismo da glicose e da insulina e resistência à insulina, levando ao aparecimento de esteatose hepática não alcoólica (NAFLD) e T2DM. Sabe-se ainda que a T2DM e a disglicémia afetam vários sistemas, como o sistema reprodutor masculino. De facto, há uma maior prevalência de subfertilidade/infertilidade em indivíduos com diabetes. Para além disso, a glicose, a insulina e os lípidos têm um papel fundamental no controlo do metabolismo testicular. Efetivamente, as células de Sertoli dependem da glicose em circulação para produzir lactato, o substrato energético usado pelas células germinativas em desenvolvimento. Assim, o metabolismo das células testiculares é crucial para a ocorrência normal de espermatogénese e qualquer desregulação pode levar à diminuição da fertilidade.

O objetivo geral deste trabalho foi o de contribuir para o entendimento da regulação homeostática do metabolismo da insulina, glicose e lípidos em estados metabólicos alterados, como obesidade e resistência à insulina. Primeiramente, avaliámos o metaboloma do fígado, músculo e tecido adiposo castanho no contexto de uma alimentação com alto teor de gordura ou frutose durante 12 semanas, abrangendo a fase equivalente à puberdade e à idade adulta. Os nossos resultados mostram que uma dieta rica em gordura durante 12 semanas causou aumento do peso, intolerância à glicose e resistência à insulina, enquanto a dieta rica em frutose durante o mesmo período de tempo levou à diminuição do peso corporal e não causou alterações na tolerância à glicose. Embora as dietas tenham efeitos distintos no ganho de peso corporal e tolerância à glicose, ambas causaram alterações significativas no metaboloma dos tecidos. No entanto, a dieta rica em gordura afetou de modo mais pronunciado o metaboloma do fígado e do músculo, enquanto a dieta rica em frutose afectou mais o metaboloma do tecido adiposo castanho. Além disso, ambas as dietas levaram a uma diminuição significativa na expressão de PON1. Tendo isso em conta, avaliámos o impacto da deleção de *Pon1*

nas consequências metabólicas da ingestão de uma dieta normal, dieta rica em gordura ou dieta rica em frutose. Neste estudo, a deleção de *Pon1* levou ao aumento da intolerância à glicose em murganhos alimentados com dieta normal ou com uma dieta rica em gordura. Além disso, a ausência de PON1 levou ao aumento do péptido-C sem alterações nos níveis de insulina em todas as dietas, o que aponta para uma diminuição da *clearance* da insulina ou diminuição da excreção do péptido-C. Em termos de deposição lipídica no fígado, não foram encontradas diferenças por conta da deleção de *Pon1*, independentemente da dieta. Considerando os resultados anteriores, foi realizada uma análise de *clusters* em indíviduos normoglicémicos, com pré-diabetes ou T2DM para verificar se a atividade paraoxonase da PON1 (POase) e os níveis de apolipoproteína J poderiam melhorar o diagnóstico de dismetabolismo, ajudando a refinar e desvendar *clusters* com significado fisiopatológico distinto. Esta análise de *clusters* identificou quatro grupos. Um deles agrupou os indivíduos jovens e aparentemente saudáveis e, portanto, sem disglicémia nem dislipidémia e com alta POase. Outros indivíduos foram agrupados num grupo maioritariamente caracterizado por disglicémia, hiperinsulinémia e dislipidémia. Os restantes grupos apresentaram perfis intermédios de insulina e glicose em comparação com os dois primeiros grupos. No entanto, estes grupos apenas se distinguiam entre si pela POase. Sendo assim, a nossa hipótese é de que esses indivíduos com menor atividade da POase mas sem disglicémia significativa, tenham uma de duas condições: i) polimorfismos de *Pon1* que resultam em baixa atividade POase e / ou ii) doença renal, resultando em disfunção do colesterol HDL, representada por baixa atividade de POase, sendo que esta última vai de encontro aos resultados obtidos no modelo animal com a deleção de *Pon1*.

Embora se saiba que a desregulação da insulina e da glicose afetam a função reprodutiva masculina, os mecanismos exatos permanecem amplamente desconhecidos. Assim, avaliámos o impacto da hiperinsulinémia na função reprodutiva masculina. Para isso, usamos um modelo animal com deleção do gene que codifica para a enzima degradadora da insulina (IDE), levando a que os animais desenvolvam hiperinsulinémia. Os nossos resultados mostram que murganhos sem IDE apresentam uma morfologia testicular comprometida. Para além disso, estes animais também apresentam uma redução na qualidade espermática, nomeadamente da viabilidade e alterações na morfologia dos espermatozoides. Assim, este estudo mostra que a IDE desempenha um papel importante na determinação do potencial reprodutivo masculino.

Em conclusão, este trabalho demonstrou que dietas ricas em gorduras e em frutose têm um impacto diferente, não apenas homeostase da glicose e da insulina, mas também nos tecidos sensíveis à insulina. Para além disso, os níveis hepáticos de PON1 foram afetados por essas dietas. Usando um modelo animal geneticamente modificado, foi demonstrado que a PON1 desempenha um papel na homeostase sistémica da glicose, sem afetar o ganho de peso. Após uma análise de *clusters* utilizando a atividade da POase, juntamente com o perfil lipídico e a função renal para informar a análise, verificou-se que a atividade da POase pode ser um valioso co-biomarcador para refinar perfis dismetabólicos com um significado fisiopatológico possivelmente distinto. Por fim, a IDE, por ter um papel na homeostase da insulina, contribui para a regulação da função reprodutiva masculina.

Agrupados, os estudos que compõem esta dissertação demonstram que a PON1 e a IDE desempenham um papel na patogénese das co-morbilidades associadas à obesidade à pré-diabetes.

Palavras-chave: Pré-diabetes; Doença do Fígado Gordo Não Alcoólico; Fertilidade Masculina; Insulino-resistência; Dieta rica em lípidos; Dieta rica em frutose; Paraoxonase-1; Apolipoproteína J; Enzima degradadora da insulina.

ABSTRACT

In the last decades the worldwide prevalence of metabolic diseases, as obesity and type 2 diabetes mellitus (T2DM), has exponentially increase posing as a serious health, social and economic concern. This increase parallels the increase of sedentarism and overnutrition. In fact, industrialization and urbanization led to the increased consumption of foods with high-carbohydrate and fat content. These, along with genetic predisposition, are the foremost contributors to metabolic dysregulation, namely hyperglycemia, dyslipidemia, and insulin resistance (IR), which ultimately lead to the onset of metabolic complications in several organs, such as the liver, muscle, adipose tissue and testes.

The liver has a key role for the regulation of whole-body glucose homeostasis. In the fed state, liver uptakes and converts circulating glucose into glycogen. On the other hand, in the fasted state, glucose is produced in from stored glycogen to maintain circulating glucose levels within normal ranges. Indeed, both glucose and insulin whole-body homeostasis are tightly regulated by a neuronal and hormonal network. Insulin has a pivotal role in controlling hepatic metabolism, by stimulating glycolysis and lipogenesis while suppressing endogenous glucose production and ketogenesis. Any imbalance in the liver energy metabolism is a major contributor to overall glucose and insulin dysmetabolism, insulin resistance, and ectopic lipid accumulation ultimately leading to the onset of non-alcoholic fatty liver disease (NAFLD) and T2DM. T2DM and dysglycemia are known to affect several systems, as is the case of male reproductive system. Indeed, there is a higher prevalence of subfertility/infertility in individuals with diabetes. In fact, glucose, insulin and lipids have a key role in controlling the testicular metabolism. Moreover, Sertoli cells depend on circulating glucose to produce lactate, the energetic substrate used by developing germ cells. Thus, the metabolism of testicular cells is crucial for the normal occurrence of spermatogenesis and any dysregulation may lead to decreased fertility.

The overall goal of this research work was to contribute to the understanding of the homeostatic regulation of insulin, glucose and lipid metabolism in altered metabolic states such as obesity and IR.

Firstly, we assessed the metabolome of liver, muscle and brown adipose tissue in the context of high-fat or high-fructose feeding for 12 weeks, covering the phase equivalent to puberty and adulthood. Our results demonstrate that a 12-week high-fat diet caused increased weight gain, glucose intolerance and insulin resistance, whereas high-fructose led to decreased body weight and did not lead to alterations in glucose tolerance. Although the diets had distinct effects on body weight gain and glucose tolerance, both led to significant alterations in the tissue's metabolome. However, high-fat diet affected more the metabolome of liver and muscle, while high-fructose diet seemed to affect more the brown adipose tissue. Moreover, both high-fat and high-fructose diet caused a significant decrease in the hepatic protein levels of PON1. Thus, the impact of whole-body *Pon1* deletion upon the intake of normal chow, high-fat or high-fructose diets was evaluated. In this study, *Pon1* deletion led to increased glucose intolerance in mice fed with normal chow diet or high-fat diet. Moreover, the absence of PON1 led to increased C-peptide levels with no changes in insulin levels independently

of the diet, which points towards either decreased insulin clearance or impaired C-peptide excretion. In terms of ectopic lipid deposition, no differences where found upon *Pon1* deletion regardless of the diet. Considering the previous results, a cluster analysis was performed in individuals with normoglycemia, prediabetes or T2DM, to evaluate whether PON1 paraoxonase (POase) activity and apolipoprotein J levels could improve the diagnosis of dysmetabolism, helping to refine and unveil clusters with distinct pathophysiological significance. In fact, the cluster analysis identified four clusters. One with the considered healthy and young individuals, and thus without dysglycemia, dyslipidemia and with high POase. Other cluster grouped individuals with dysglycemia, hyperinsulinemia, and dyslipidemia. The other two clusters presented intermediate insulin and glucose profiles compared with the first two clusters. However, these two groups were distinct in relation to POase activity. We hypothesize that these individuals with lower POase activity but without significant dysglycemia have one of two conditions: i) PON1 polymorphisms that result in low POase activity and/or ii) kidney disease, resulting in high-density lipoprotein cholesterol dysfunction that is represented by low POase activity. This last hypothesis goes in accordance with the results of *Pon1* knockout model.

Although it is known that insulin ang glucose dysregulation affects male reproductive function, the exact mechanisms by which insulin dysfunction mediates these effects remain largely unknown. Thus, we assessed the impact of primary hyperinsulinemia on male reproductive function. For that, we used a whole-body knockout model for *Ide*, the insulin-degrading enzyme (IDE). In the absence of IDE, insulin is not degraded in the liver, leading to hyperinsulinemia. Our results show that knockout mice for *Ide* mice present impaired testicular morphology. Moreover, these mice also present impaired sperm quality, namely a decrease in both sperm viability and morphology. Thus, this study provides evidence that IDE plays an important role in determining the reproductive potential of males.

In conclusion, this research work demonstrated that high-fat and high-fructose diets have a different impact not only in overall glucose and insulin homeostasis, but also on insulin-sensitive tissues. Moreover, hepatic PON1 levels were found to be affected by these diets. By using a genetically modified mouse model, it was demonstrated that PON1 plays a role in glucose homeostasis, without affecting weight gain. After performing a cluster analysis in individuals with normoglycemia, prediabetes or T2DM, using POase activity along with lipid profile and renal function to inform the analysis, it was found that POase activity might be a valuable co-biomarker to refine dysmetabolic profiles with potentially different pathophysiological meaning. Lastly, IDE, by having a role in insulin homeostasis, contributes to a normal male reproductive function. Together, the individual studies that compose this dissertation demonstrate that PON1 and IDE play a role in the pathogenesis of obesity and prediabetes-associated co-morbidities.

Keywords: Prediabetes; Non-Alcoholic Fatty Liver Disease; Male Fertility; Insulin Resistance; High-Fat Diet; High-Fructose Diet; Paraoxonase-1; Apolipoprotein J; Insulin-Degrading Enzyme.

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^{1}H-NMR	Proton Nuclear Magnetic Resonance
3-OH-butyrate	3-hydroxybutyrate
4-HNE	4-hydroxynonenal
a.u.	arbitrary units
AASLD	American Association for the Study of Liver Diseases
ACC	Acetyl-Coenzyme A carboxylase
Acetyl-CoA	Acetyl-Coenzyme A
ACL	Adenosine triphosphate-citrate lyase
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
APDP	Portuguese Diabetes Association
ApoAI	Apolipoprotein A-I
ApoJ	Apolipoprotein J
AQP	Aquaporin
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ATP	Adenosine triphosphate
AUC	Area under the curve
BAT	Brown adipose tissue
Bax	B-cell lymphoma 2-associated protein
BCA	Bicinchoninic acid
BCAA	Branched-chain amino acids
Bcl-2	B-cell lymphoma 2
BMI	Body mass index
BW	Body weight
cAMP	Cyclic adenosine monophosphate
Casp3	Caspase 3
CD36	Cluster of differentiation 36
cDNA	Complementary deoxyribonucleic acid
CEACAM1	Carcinoembryonic antigen–related cell adhesion molecule 1
ChoRE	Carbohydrate response element
ChREBP	Carbohydrate response element binding protein
CLU	Clusterin
CoA	Coenzyme A

CPT1	Carnitine palmitoyl transferase 1
CVD	Cardiovascular disease
DM	Diabetes *mellitus*
DNA	Deoxyribonucleic Acid
eGFR	Estimated glomerular filtration rate
ELOVL2	Long chain fatty acid elongase 2
EMA	European Medicines Agency
EMMA	The European Mouse Mutant Archives
F1,6Pase	Fructose-1,6-bisphosphatase
FA	Fatty acids
$FADH_2$	Flavin adenine dinucleotide, reduced form
FAS	Fatty acid synthase
FDA	Food and Drug Administration
FFA	Free fatty acids
FLI	Fatty liver index
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GK	Glucokinase
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GLUTs	Glucose transporters
GSH	Glutathione
GSI	Gonadosomatic index
HbA1c	Glycated hemoglobin
HCC	Hepatocellular carcinoma
HDL	High-density lipoproteins
HDL-c	High-density lipoprotein cholesterol
Het	Heterozygous
HFat	High-fat
HFruct	High-fructose
HPLC	High performance liquid chromatography
IDE	Insulin-degrading enzyme
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGF-1	Insulin-like growth factor 1
IGT	Impaired glucose tolerance
IL-6	Interleukin-6
IMP	Inosine monophosphate

InsR	Insulin receptor
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
KO	Knockout
LDL	Low-density lipoproteins
LDL-c	Low-density lipoprotein cholesterol
LXR	Liver X receptor
LXRα	Liver X receptor α
MCP-1	Monocyte chemoattractant protein-1
mRNA	Messenger ribonucleic acid
MTBE	Methyl-tert-butyl ether
mtDNA	Mitochondrial DNA
MUFA	Monounsaturated fatty acids
N.A.	Non-applicable
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAFLD-FLS	Non-alcoholic fatty liver disease - fatty liver score
NASH	Non-alcoholic steatohepatitis
NCD	Normal chow diet
nCLU	Nuclear clusterin
NF-kB	Nuclear factor kappa B
oxLDL	Oxidized low-density lipoprotein
OXPHOS	Oxidative phosphorylation
p38MAPK	P38 mitogen-activated protein kinases
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxylase
PFK1	Phosphofructokinase 1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
PI3K	Phosphoinositide-3 kinase
PKA	Protein kinase A
PLS-DA	Partial least squares discriminant analysis
POase	Paraoxonase activity
PON	Paraoxonase
PON1	Paraoxonase-1
PON2	Paraoxonase-2
PON3	Paraoxonase-3

PPAR	Peroxisome proliferator-activated receptors
PPARα	Peroxisome proliferator-activated receptor α
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCD1	Stearoyl-CoA desaturase 1
sCLU	Secretory clusterin
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SHBG	Sex hormone-binding globulin
SNPs	Single nucleotide polymorphisms
Sp1	Specificity protein 1
SRE	Sterol regulatory element
SREBP1c	Sterol regulatory element-binding protein 1c
SREBP2	Sterol regulatory element-binding protein 2
T1DM	Type 1 diabetes *mellitus*
T2DM	Type 2 diabetes *mellitus*
TAG	Triglycerides
TCA	Tricarboxylic acid
TNFα	Tumor necrosis factor α
TOCSY	Total correlated spectroscopy
tRNA	total RNA
UDPG	Uridine diphosphate glucose
UTR	Untranslated region
VIP	Variable importance in the projection
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
WHO	World Health Organization
WT	Wildtype
α-KG	α-ketoglutarate
ß2M	ß-2 microglobulin

I. INTRODUCTION

This chapter was adapted from the published works:

Meneses MJ, Silvestre R, Sousa-Lima I, Macedo MP (2019). Paraoxonase-1 as a Regulator of Glucose and Lipid Homeostasis: Impact on the Onset and Progression of Metabolic Disorders. International Journal of Molecular Sciences. 20(16):4049.

Meneses MJ (2017). Metabolic Disorders and Male Reproductive Health. In: Biochemistry of Andrology. Editors: Marco G. Alves and Pedro F. Oliveira. Bentham Science. (ISBN:978-1-68108-501-2).

1. Obesity, Prediabetes/Type 2 Diabetes *mellitus* and Associated Pathologies

In the last decades metabolic diseases, namely obesity and type 2 diabetes mellitus (T2DM), have spread worldwide becoming a serious health and social concern. The exponential increase in the prevalence of these diseases parallels the increase of sedentarism and overnutrition. In fact, industrialization and urbanization led to the increased consumption of foods with high-carbohydrate and fat content. These, along with genetic factors, are major contributors to metabolic dysregulation, namely hyperglycemia, dyslipidemia, and insulin resistance (IR), which will ultimately lead to the onset of metabolic complications in several organs and systems.

Obesity

Obesity is defined by the World Health Organization (WHO) as abnormal or excessive fat accumulation. Although the awareness for the impact of obesity has increased in the last years, its prevalence continues to rise dramatically. In fact, and comparing to 1975, the prevalence of obesity worldwide has nearly tripled and more than 60% of adults in western countries are overweight (Nguyen and El-Serag, 2010); by 2025, it is expected that global obesity prevalence will reach 18% in men and exceed 21% in women. Moreover, severe obesity will surpass 6% in men and 9% in women (NCD Risk Factor Collaboration, 2016). In 2015, a study demonstrated that the prevalence of obesity and overweight in Portugal was of 22.3% and 34.8%, respectively (Oliveira *et al.*, 2018). There are several factors that may be implicated in the etiology of obesity, namely genetics, environment and socioeconomic and psychological factors (Rashid *et al.*, 2003). Although genetic background influences the development of obesity (Grarup *et al.*, 2014), the vast majority of obesity cases are due to factors that arose in western countries, namely the excessive consumption of highly-processed and energy dense foods, which are frequently combined with a sedentary lifestyle. The most used method to classify obesity is the body mass index (BMI). BMI is calculated through the ratio between the weight (kg) and the squared height (m^2) (Ng *et al.*, 2014). An individual is considered overweight when the BMI is higher than 25 kg/m^2 but lower than 30 kg/m^2. When BMI exceeds 30 kg/m^2 the individual is considered obese; obesity may be further classified in morbid obesity when BMI surpasses 40 kg/m^2. However, and as body fat distribution is a risk factor for the development of comorbidities, other measures such as waist circumference or waist-to-hip circumference may be used in the clinical practice, to refine the diagnosis obtained through BMI (Klein *et al.*, 2007). In fact, abdominal fat is associated with increased metabolic risk factors and increased risk of cardiometabolic disease (Grundy, 2004, Klein *et al.*, 2007). Undeniably, obesity causes numerous abnormalities in lipid metabolism, including increased plasma triglycerides, lower levels of circulating high density lipoprotein cholesterol (HDL-c) and alterations in lipoprotein structure and concentrations, namely due to dysregulated production of adipokines (Furukawa *et al.*, 2004, Kota *et al.*, 2013, Mutlu-Turkoglu *et al.*, 2003). Adipose tissue also secretes non-esterified fatty acids to the bloodstream, and their flux to the liver may affect liver metabolism, increasing hepatic IR and glucose production (Bergman *et al.*, 2006). Moreover,

proinflammatory cytokines are also secreted resulting in low-grade inflammation that leads to IR and vascular dysfunction (Kim *et al.*, 2008, Rask-Madsen and Kahn, 2012). Besides the increased risk of T2DM, obesity is linked to other health problems, including non-alcoholic fatty liver disease (NAFLD), infertility, atherosclerosis, degenerative disorders and several types of cancer (Semenkovich, 2006).

Prediabetes and Type 2 Diabetes *mellitus*

Diabetes *mellitus* (DM) is a metabolic and chronic disease, considered one of the leading causes of morbidity and mortality worldwide. This burden is having such an exponential increase that statistical projections become outdated years ahead of schedule. One of the most recent studies by the International Diabetes Federation (IDF) estimates that 463 million adults (20-79 years) have DM and that this number will rise to 578 million by 2030 (International Diabetes Federation, 2019). In Portugal, the estimated prevalence of DM in individuals between 20 and 79 years old was, in 2018, of 13.6% (Raposo, 2020). DM is characterized by hyperglycemia due to inadequate pancreatic ß-cells insulin production, to poor response to insulin, or both (American Diabetes Association, 2015). In addition, DM causes impaired carbohydrate, fat, and protein metabolism (Tay *et al.*, 2015). There are two main types of DM: type 1 DM (T1DM) and T2DM. The first one is an autoimmune disease that leads to the destruction of insulin-producing ß-cells (Alam *et al.*, 2014). Consequently, there is a dramatic reduction or even ablation of insulin production and patients need exogenous insulin to survive therefore being also called insulin-dependent DM (Xia *et al.*, 2018). On the other hand, hyperglycemia in T2DM results from IR along with variable degrees of inadequate insulin secretion and/or clearance (Alam *et al.*, 2014, Najjar and Perdomo, 2019)(Figure 1). Contrary to T1DM, the key triggers for T2DM are unhealthy eating habits and sedentarism, although genetic factors may also play a role (Merino *et al.*, 2017). The complexity of T2DM led to the establishment of a prodromal stage named as prediabetes or intermediate hyperglycemia (Meneses *et al.*, 2015a, Tabak *et al.*, 2012).

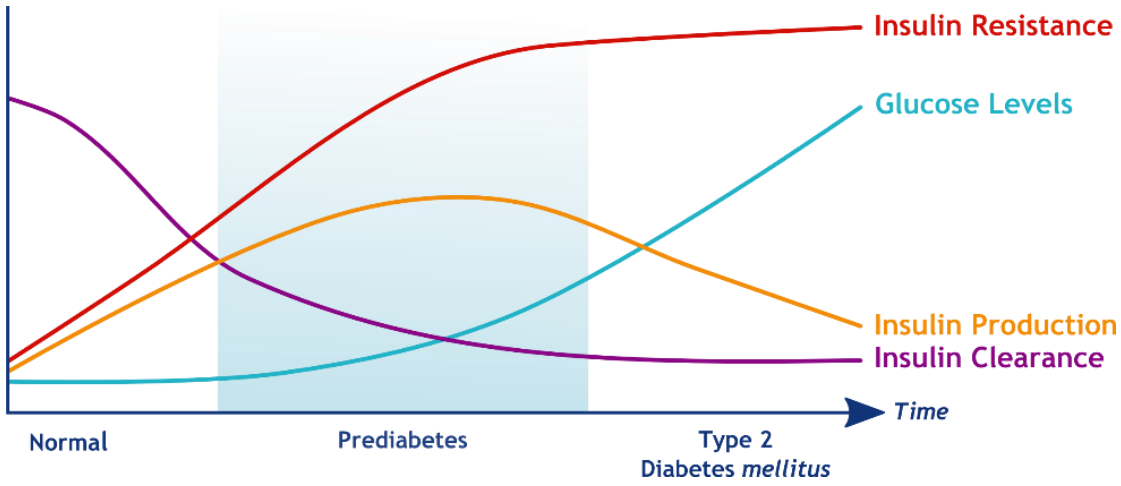

Figure 1 – Natural history of type 2 diabetes *mellitus*. The development and progression of type 2 diabetes *mellitus* begins with a fully compensated insulin-resistant state, where insulin levels rise and insulin clearance decreases to maintain glycemia within normal values, progressing to prediabetes. Later, ß-cells become dysfunctional and unable to fully compensate insulin resistance, leading to type 2 diabetes *mellitus*.

Prediabetes is the most important predictor of T2DM and is characterized by increased glucose levels, although lower than the diabetes threshold. It is characterized by impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) (de Vegt *et al.*, 2001). Similar to the name given to this state (prediabetes vs. intermediate hyperglycemia), the threshold glucose levels and the diagnostic criteria also vary between organizations (Table 1).

Table 1 – Current diagnostic criteria for prediabetes according to different organizations.

	State	IDF/WHO	ADA
IFG	Fasting	110-125 mg/dL	100-125 mg/dL
	2 h after OGTT	<140 mg/dL	<140 mg/dL
	HbA_{1c}	N.A.	5.7-6.4%
IGT	Fasting	<110 mg/dL	<100 mg/dL
	2 h after OGTT	140-199 mg/dL	140-199 mg/dL
	HbA_{1c}	N.A.	5.7-6.4%
IFG+IGT	Fasting	110-125 mg/dL	100-125 mg/dL
	2 h after OGTT	140-199 mg/dL	140-199 mg/dL
	HbA_{1c}	N.A.	5.7-6.4%

IDF – International Diabetes Federation; WHO – World Health Organization; ADA – American Diabetes Association; IFG – Impaired Fasting Glucose; IGT – Impaired Glucose Tolerance; OGTT – Oral Glucose Tolerance Test; HbA_{1c} – glycated hemoglobin; N.A. – non-applicable.

Due to the heterogeneity of prediabetes, several studies have evaluated individuals with isolated IGT or IFG to better understand their predictive value in the progression to T2DM. While IFG appears to be more connected to impaired insulin secretion and progressive increase in hepatic glucose production, IGT appears to be linked to lower insulin sensitivity and secondary impairment of insulin secretion. However, both states are characterized by hyperglycemia as the mechanisms are not totally independent (Faerch *et al.*, 2016, Stefan *et al.*, 2016). Prediabetes was initially thought to be an innocuous condition. More importantly, besides the plasma glucose levels other *milieu* molecules are drivers of pathological conditions associated with the progress of T2DM. Conversely, nowadays it is known that several associated pathologies, that were considered only in the presence of established diabetes, as is the case of micro and macrovascular complications, non-alcoholic fatty liver disease and subfertility, may appear along with prediabetes onset on the basis of altered glucose, lipids and other *milieu* molecules (Buysschaert *et al.*, 2015, Tabak *et al.*, 2012).

Hallmarks of Metabolic Disorders

Cellular homeostasis and an overall balanced metabolic system are crucial requirements for health. Any dysregulation in this balance may lead to the onset and progression of metabolic disorders. These, namely obesity and prediabetes/T2DM, are characterized by an overall state of inflammation and

oxidation that contributes to the onset of IR. In fact, all these conditions will interplay with several molecular pathways creating a vicious cycle of metabolic impairment.

Inflammation

Inflammation is a protective and complex biological response of the organism to noxious stimuli. It can be considered acute or chronic with the latter being associated with metabolic, cardiovascular and neurodegenerative diseases (Okin and Medzhitov, 2012). It leads to the induction of repair mechanisms in response to any alteration in order to restore tissue homeostasis. Although chronic subclinical inflammation is considered a hallmark of metabolic disorders, the events that trigger the inflammatory process are not fully understood. Inflammation is characterized by the secretion of inflammatory inducers. These inducers may be adipokines and chemokines, as is the case of interleukin-6 (IL-6), tumor necrosis factor α (TNFα) and monocyte chemoattractant protein-1 (MCP-1) or even advanced glycation end products (AGEs), oxidized lipoproteins, and microbial inducers (Medzhitov, 2010). These molecules will then trigger the inflammatory response. Moreover, they may then act in both autocrine and paracrine manner and induce IR by activating c-JUN N-terminal kinase (JNK) and nuclear factor-kappa B (NF-kB) pathways, known to promote tissue inflammation (Esser *et al.*, 2014).

Oxidative stress

Oxidative stress results from the imbalance between antioxidant defenses and oxidative stress, in favor of the latter, leading to increased intracellular levels of reactive oxygen species (ROS). ROS are byproducts of oxygen metabolism and energy production (Bayr, 2005). This broad term includes both oxygen radicals and certain non-radical oxidizing agents that can be easily converted into radicals. ROS are normally produced in cells, playing important roles in cell signaling (O'Flaherty *et al.*, 2006). However, when produced over the antioxidant capacity of the cell, ROS lead to the disruption of redox signaling and to molecular damage, which can ultimately result in cell death (Bisbal *et al.*, 2010, Sies, 2015). Thus, oxidative stress plays an important role in inflammatory and metabolic disorders (Bisbal *et al.*, 2010, Kota *et al.*, 2013). In fact, oxidative stress was already found to suppress several steps of the insulin receptor signal transduction, leading to decreased expression of glucose transporter 4 (GLUT4) in the plasma membrane, thus being a contributor for IR in the peripheral tissues (Hurrle and Hsu, 2017).

ß-cell dysfunction

ß-cells synthetize, store and secrete insulin in response to multiple and integrated signals. ß-cell dysfunction is a key process to the onset and progression of diabetes, as a result of the interplay between genetic and lifestyle factors, acting via several pathophysiological mechanisms (Poitout and Robertson, 2008, Prentki *et al.*, 2020). Although other physiological molecules regulate insulin secretion, as amino acids, hormones and ketones, the most important regulator of insulin release is glucose (Marchetti *et al.*, 2020). Glucose enters the cytoplasm of ß-cells through glucose transporters

(GLUTs), mainly glucose transporter 1 (GLUT1). It then undergoes glycolysis to form pyruvate. As the expression of lactate dehydrogenase, the enzyme that converts pyruvate into lactate, is very low, pyruvate is directed to the mitochondria to enter in the tricarboxylic acid cycle (TCA or Krebs cycle). Nicotinamide adenine dinucleotide reduced (NADH) and flavin adenine dinucleotide reduced ($FADH_2$), resultant from the TCA cycle, are used by the electron transporter chain to generate adenosine triphosphate (ATP) (Maechler, 2013). The translocation of ATP to the cytosol and the consequent increase in ATP/ adenosine diphosphate (ADP) ratio leads to the closure of ATP-sensitive potassium channels. This causes depolarization of the plasma membrane, resulting in the opening of voltage-dependent calcium channels, allowing calcium influx into the ß-cell. The increase in intracellular calcium concentration leads to the fusion of the plasma membrane with insulin secretory granules, releasing the insulin to the circulation (Maechler, 2013, Marchetti *et al.*, 2020).

Although during a short-term period, ß-cell is able to compensate an increase in glucose levels with increased insulin secretion, with time it becomes dysfunctional (Prentki *et al.*, 2020). In fact, when glucose is available in excess in the ß-cell, it is directed to alternative pathways and ROS can be formed (Poitout and Robertson, 2008). As the ß-cell has very low levels of antioxidant enzymes, excessive ROS levels may be present, ultimately leading to ß-cell dysfunction. Moreover, the first-phase of insulin release is abrogated and even the second-phase is affected in its amplitude (Thurmond and Gaisano, 2020). This is a result of the decreased expression of GLUTs and glucokinase, thus affecting glucose internalization and metabolism (Del Guerra *et al.*, 2005). Besides, the expression of mitochondrial enzymes is also altered, resulting in a decreased ATP/ADP ratio (Anello *et al.*, 2005). Altogether, these alterations lead to decreased insulin secretion.

Insulin Clearance

Insulin clearance is the process by which insulin is degraded and thus, cleared from circulation. It occurs in several organs, but mainly in the liver and kidney (Polidori *et al.*, 2016). Following its pulsatile secretion (Laurenti *et al.*, 2019), insulin is transported via the portal vein and reaches the hepatocytes after crossing the fenestrations in the liver sinusoidal endothelium (Mohamad *et al.*, 2016). During this first-pass, up to 70% of the insulin is cleared by the liver (Field, 1973), thus being responsible for regulating the amount of insulin that reaches peripheral organs.

The hepatic insulin clearance process begins with insulin binding to its receptor in the membrane of the hepatocytes. Insulin receptor (InsR) then recruits carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1), a protein that facilitates InsR internalization. After internalization of the complex InsR-Insulin-CEACAM1, insulin-degrading enzyme (IDE) in endosomes begins to degrade insulin, before acidification. As endosomes acidify, any partially degraded insulin will dissociate from the InsR, that may be recycled back to the membrane or translocated to lysosomes for degradation. Any remaining insulin fragments are then delivered to lysosomes for their complete proteolytic degradation (Najjar and Perdomo, 2019, Tokarz *et al.*, 2018). Insulin that is not cleared in the first pass, exits the liver through hepatic vein, reaches the heart and it is then delivered to its target

tissues. This includes the liver, where insulin is again cleared. However, insulin also acts in the liver to suppress gluconeogenesis and glycogenolysis (Rui, 2014).

Although insulin clearance is essential to, along with insulin secretion, regulate and maintain insulin levels within a normal range, it is usually disregarded. Insulin clearance may be reduced in order to increase insulin levels and to limit the need of increasing insulin release by ß-cells (Bonora *et al.*, 1983). On the other hand, impaired insulin clearance can lead to hyperinsulinemia-induced IR (Bojsen-Møller *et al.*, 2018). In fact, it has been shown that deletion of IDE (Borges *et al.*, 2019) or CEACAM1 (DeAngelis *et al.*, 2008) results in chronic hyperinsulinemia, due to impaired insulin clearance.

Insulin Resistance

Insulin is released from secretory granules in pancreatic ß-cells in response to increasing plasma levels of glucose and amino acids, in order to normalize them to physiologic levels (Weiss *et al.*, 2000). IR refers to a state where insulin-sensitive cells do not respond appropriately to circulating insulin (Macedo *et al.*, 2014, Samuel and Shulman, 2016). To overcome the reduced efficiency in insulin action, pancreatic ß-cells increase hormone release, in order to maintain normal blood glucose levels; however, this is accompanied by a chronic hyperinsulinemia which characterizes specific prediabetes and T2DM phenotypes (Kahn *et al.*, 2006). As mentioned, the liver has an important role in insulin clearance (Field, 1973). Consequently, defects in hepatic insulin clearance may also result in hyperinsulinemia (Najjar and Perdomo, 2019). With time and IR progression, pancreatic ß-cells become unable to fully compensate the decreased insulin sensitivity, leading to frank T2DM. This affects normal function of different organs such as the pancreas, liver, bone, testes and brain (Castro *et al.*, 2014, Kawai *et al.*, 2012), thus imprinting a transversal feature on metabolic disorders. In the case of liver disease, hepatic IR is a consequence of an impairment of the insulin signaling pathway and glucose uptake, leading to decreased glycogenesis and hyperglycemia (Gallagher *et al.*, 2010, Sesti, 2006). Furthermore, increased blood glucose levels lead to an increased production of ROS, ultimately potentiating the overall prooxidant state (LeRoith, 2002). Oxidative stress is known as a disease trigger, playing a significant role in the development of inflammation and fibrogenesis (Haldar *et al.*, 2015).

Dyslipidemia

IR is associated with metabolic abnormalities such as hyperglycemia and dyslipidemia (Kwon and Pessin, 2013, Yki-Jarvinen, 2014). Dyslipidemia is a lipoprotein disorder, characterized by increased levels of low-density lipoprotein cholesterol (LDL-c), decreased HDL-c, and increased serum triglycerides, thus promoting the development of atherosclerosis and cardiovascular disease (CVD). Dyslipidemia is mainly driven by IR and pro-inflammatory adipokines, that may be in turn a result of a sedentary lifestyle with excessive dietary intake of saturated fat and cholesterol. After a meal, dietary triglycerides are emulsified by bile acids. After intestinal uptake, the resulting FFA are reesterified in the enterocytes to form triglycerides. The latter are then packaged in chylomicrons, which are secreted into the lymphatic system. Chylomicrons are then mainly delivered to the muscle,

that uses them for energy production, and to the adipose tissue, which stores fatty acids. However, triglycerides may be hydrolyzed by lipoprotein lipase in the circulation, to long chain fatty acids, which can deposit along blood vessels or accumulate in the tissues, which ultimately leads to atherosclerosis. The chylomicron remnants with the remaining triglycerides, are then transported to the liver and uptaken by hepatocytes (Alves-Bezerra and Cohen, 2017). In fasting conditions, lipolysis is activated and FFA are transported to the liver. However, due to IR, the white adipose tissue becomes dysfunctional, increasing lipolysis. This leads to an overload of FFAs on all organs, compromising structure and cell signaling and dysregulating local metabolism. Therefore, obesity-induced dyslipidemia may result in dysfunction of several tissues and organs, including the liver and the reproductive system.

2. Impact of Dysmetabolism on Liver Functions

The liver plays a key role in whole-body homeostasis, acting as the central organ for both lipid and glucose metabolism (Postic *et al.*, 2004). Receiving blood from the intestine through the portal vein, the liver is responsible for storing nutrients in the postprandial state and releasing them, in a controlled manner, during fasting. Furthermore, the liver metabolizes numerous nutrients and xenobiotics, and neutralizes foreign antigens being one of the most important lines of body defense. The liver also synthesizes the bile and the major proportion of the circulating proteins in the blood, including complement system proteins and albumin.

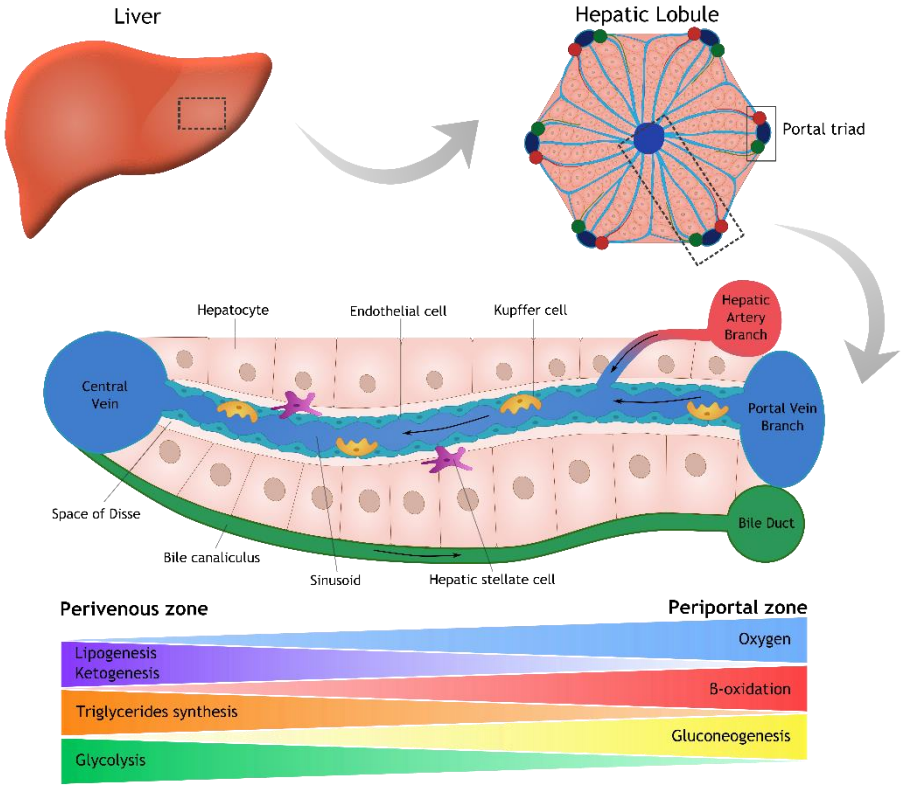

Figure 2 - Hepatic architecture. The liver is composed of hexagonal structural units, the lobules. Portal triads, located at the vertices of the lobules, comprise a branch of the hepatic artery, a branch of the portal vein and a bile duct. Blood flows through sinusoids and drains into the central vein. Non-parenchymal cells, that support hepatocytes' function, such as liver endothelial cells, Kupffer cells, and hepatic stellate cells reside along the lobule. Bile secreted from hepatocytes flows from the perivenous to the periportal zone through bile canaliculi that drain into the bile duct. The hepatocytes perform different functions according to their location in the lobule: perivenous or pericentral.

The liver is composed of parenchymal cells, the hepatocytes, that perform most of the previously described functions. Nonetheless, the liver has non-parenchymal cells: Kupffer cells, hepatic stellate cells, cholangiocytes, and sinusoidal endothelial cells (Figure 2). These cells are organized in the liver structural units, the lobules. Blood, either from the portal vein or from the hepatic artery, enters these hexagonal structures from the outer side and flows towards a central vein. On the other hand, bile acids secreted by the hepatocytes flow inside the bile canaliculi in the opposite direction to blood flow. Bile canaliculi end at bile ductules at the portal nodes, from which the bile is delivered to the

intestine. Importantly, this anatomical structure leads to a zonation, with hepatocytes performing different tasks according to their localization (Ben-Moshe and Itzkovitz, 2019)(Figure 2). The periportal hepatocytes, located in the outer side of the lobule, are well oxygenated and supplied with substrates; gluconeogenesis occurs mainly in these cells. On the other hand, perivenous hepatocytes, located in the center of the lobule, are more involved in glycolysis and ketogenesis (Rui, 2014).

Fatty Acid Metabolism

Hepatic lipid metabolism is crucial for homeostasis, being responsible for orchestrating the synthesis of fatty acids, their export and distribution to extrahepatic tissues, as well as their utilization as energy substrates. Fatty acids (FA) within the liver are obtained either from dietary or endogenous sources. Although circulating FAs can enter the hepatocytes by passive diffusion, its uptake is mostly dependent on FA transporters, namely cluster of differentiation 36 (CD36), caveolins, fatty acid-binding protein, and fatty acid transporter proteins (Mashek, 2013).

De novo *lipogenesis*

De novo lipogenesis is the process through which the liver synthesizes FAs from acetyl-coenzyme A (acetyl-CoA) subunits. These subunits, in turn, may be produced from several pathways, generally from carbohydrate catabolism (Sanders and Griffin, 2016). Although glucose is the main substrate for *de novo* lipogenesis, fructose may also be used as a substrate (Dekker *et al.*, 2010). Carbohydrates are hydrolyzed into pyruvate through glycolysis. In the mitochondria, pyruvate generates acetyl-CoA, which is in turn combined with oxaloacetate to form citrate (Jones, 2016). The latter is exported to the cytoplasm and, due to insulin action, is split into oxaloacetate and acetyl-CoA by ATP-citrate lyase (ACL). Acetyl-CoA is then converted into malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA inhibits carnitine palmitoyl transferase 1 (CPT1) thereby blocking ß-oxidation, the process of FA degradation (McGarry *et al.*, 1978) (Figure 3). FA synthase (FAS) catalyzes the formation of palmitic acid from malonyl-CoA and acetyl-CoA. After the rate-limiting step of *de novo* lipogenesis, palmitate can be modified through elongation, desaturation and reactions by long-chain fatty acid elongase 2 (ELOVL2) and stearoyl-CoA desaturase 1 (SCD1), respectively, to produce complex FAs as palmitoleic acid and oleic acid (Bessone *et al.*, 2019, Song *et al.*, 2018). These newly synthesized FAs may be esterified with glycerol-3-phosphate to produce triacylglycerol (Rui, 2014).

Genetic control of *de novo* lipogenesis

De novo lipogenesis is primarily regulated at the transcriptional level, namely by sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate response element-binding protein (ChREBP) (Oosterveer and Schoonjans, 2014). SREBP1c is postprandially activated by the increase in insulin levels. Although the exact mechanism remains to be unveiled, it is known that SREBP1c activation occurs through the phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB/Akt) pathway, leading to the phosphorylation of the SREBP1c and to the activation of the liver X receptor (LXR), mainly the LXRα isoform, a known activator of SREBP1c. ChREBP is also postprandially activated but in response to an increase in carbohydrates. The exact molecular mechanism by which ChREBP is activated is also

unclear, but it appears to be stimulated by some metabolites generated during glycolysis, as is the case of glucose-6-phosphate and fructose-2,6-bisphosphate (Arden *et al.*, 2012). After its activation, ChREBP targets genes containing carbohydrate response element, namely ACC, FAS and SCD1 (Sanders and Griffin, 2016). Moreover, ChREBP also targets liver-pyruvate kinase (L-PK), which provides pyruvate increasing the levels of substrate for *de novo* lipogenesis (Uyeda and Repa, 2006) (Figure 3).

In the fasting state, the increase in blood glucagon levels and cellular cyclic adenosine monophosphate (cAMP) levels activate adenosine monophosphate (AMP)-activated protein kinase (AMPK) and cAMP-dependent protein kinase (PKA) leading to the inhibition of *de novo* lipogenesis (Kawaguchi *et al.*, 2001, Lu and Shyy, 2006). In the fed state, only 4% of triglycerides secreted into circulation in a lean healthy individual come from *de novo* lipogenesis (Diraison and Beylot, 1998). On the other side, in obese and insulin-resistant individuals, this percentage rises to 25-30% of the total triglyceride content (Leavens and Birnbaum, 2011).

ß-oxidation

ß-oxidation is the process through which FAs are degraded into acetyl-CoA to synthesize ATP and ketone bodies. Although ß-oxidation mostly occurs in the mitochondria, it may take place on peroxisomes, as is the case of very long-chain FAs and in the endoplasmatic reticulum. While short and medium-chain FAs cross the membranes without the need for activation, long-chain FA need to be activated by acyl-CoA synthetase to acyl-CoA. Afterward, they are transported across the mitochondrial membrane by CPT1 (Geisler and Renquist, 2017). Here, acyl-CoAs are degraded via ß-oxidation, leading to the formation of a two-carbon chain–shortened acyl-CoA and to acetyl-CoA (Houten *et al.*, 2016). The latter are further oxidized in the TCA cycle to produce NADH and $FADH_2$ for ATP synthesis by the electron transport chain. When several acetyl-CoA molecules are present, they are processed into ketone bodies (McGarry and Foster, 1980) (Figure 3).

FA ß-oxidation rates are higher in the fasting state, with peroxisome proliferator-activated receptor α (PPARα) acting as a potent positive modulator of this pathway. PPARα is activated by long-chain FA, and upregulates the expression of genes involved in both mitochondrial and peroxisomal ß-oxidation (Berlanga *et al.*, 2014, Rui, 2014). In the fed state, ß-oxidation is inhibited, in part due to the inhibition of CPT1 by malonyl-CoA (Rui, 2014).

As explained, there is a strict network of activators and repressors of both lipid synthesis and oxidation to guarantee the physiological level of hepatic triglycerides and avoid a loop of synthesis and oxidation. However, in a state of IR, there is a disruption in this balance, consequence of molecular events that promote an increase in hepatic triglyceride accumulation that lead to steatosis (Bessone *et al.*, 2019). The detailed understanding of the molecular mechanisms driving the fat accumulation and oxidative imbalance is essential for the diagnosis and treatment of NAFLD.

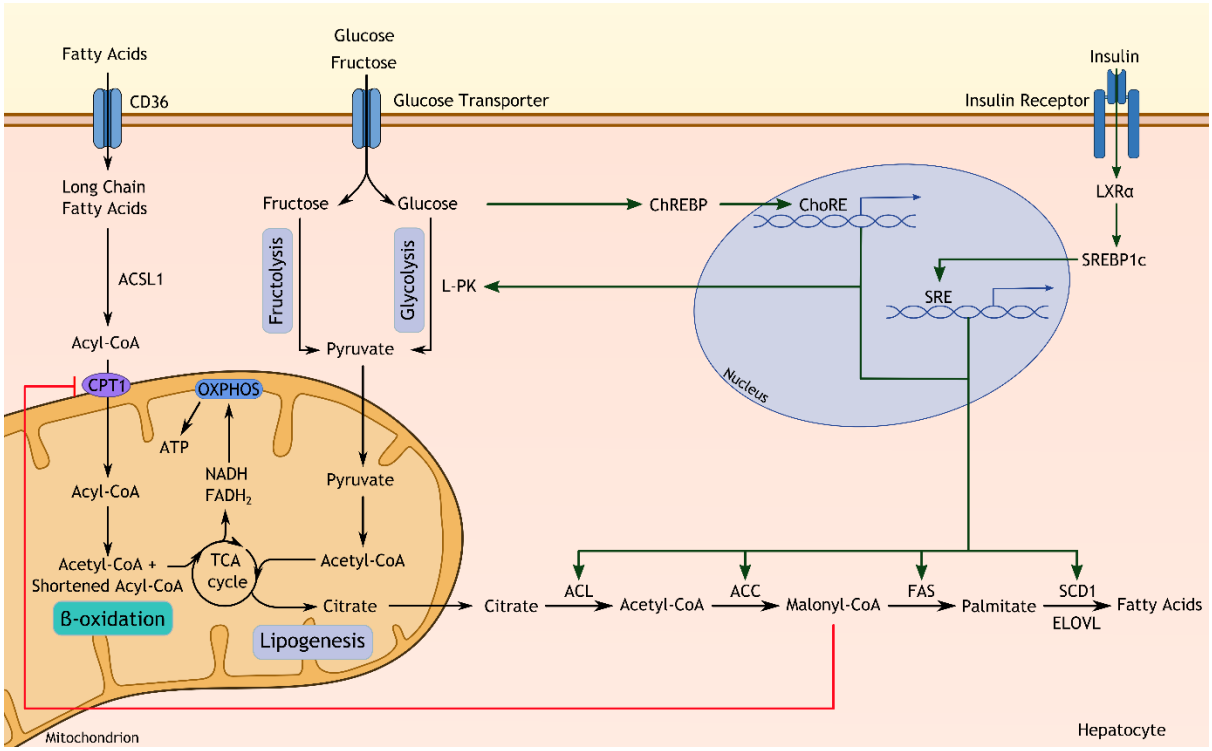

Figure 3 – *De novo* lipogenesis and β-oxidation. In the liver, insulin induces SREBP1c expression, leading to the transcriptional activation of lipogenic genes. Simultaneously, glucose activates ChREBP, which transcriptionally activates L-PK and lipogenic genes. The synergistic actions of SREBP1c and ChREBP activate the enzymatic machinery necessary for the conversion of excess glucose and fructose to fatty acids. The increased fatty acid synthesis leads to the increased production of malonyl-CoA, which inhibits CPT1, therefore inhibiting fatty acid transport into the mitochondria and inhibiting β-oxidation. In fasting and insulin resistant states, the free fatty acids that are uptaken by the liver from the periphery will be processed to produce acetyl-CoA. ACC - Acetyl-CoA carboxylase; ACL - ATP citrate lyase; ACSL1 - Long-chain-fatty-acid-CoA ligase 1; CD36 – cluster of differentiation 36; ChoRE - carbohydrate response element; CPT1 - carnitine palmitoyl transferase-1; ELOVL - elongation of long chain fatty acids; FAS - fatty acid synthase; L-PK – Liver pyruvate kinase; LXRα – liver X receptor α; SCD1 - stearoyl-CoA desaturase; SRE - Sterol regulatory element; SREBP1c - sterol regulatory element-binding protein 1c. → activation; ⊣ inhibition

Glucose and Fructose Metabolism

As is the case for lipid metabolism, the liver plays a critical role in controlling carbohydrate metabolism (Jones, 2016). The process of either carbohydrate breakdown or synthesis in hepatocytes is under a tight control with insulin playing a key role. Even though historically glucose metabolism has been given more importance, we cannot disregard fructose metabolism. Although these carbohydrates' have the same chemical formula, there are small differences in their chemical structure that make their metabolism unique. First, while blood glucose levels rise after a meal, fructose blood levels remain fairly constant, as fructose is almost completely removed upon first-pass in the liver (Mayes, 1993, Rippe and Angelopoulos, 2013). Then, as fructose metabolism is mostly independent on insulin secretion, the first steps of its metabolism also differ. In fact, these were the major reasons for the initial proposal that fructose could be a natural substitute of sucrose for patients with DM (Tappy *et al.*, 2010). Although naturally occurring, free fructose corresponds to a small component of total energy intake, the increased consumption of sweeteners in the last decades,

namely high-fructose corn syrup, contributed to an important increase in overall fructose consumption (Vos *et al.*, 2008). Besides, the increased consumption of carbohydrates leads to changes in hepatic carbohydrate metabolism causing IR, increased hepatic glucose production and *de novo* lipogenesis, oxidative stress and low-grade inflammation. Knowledge of the metabolic processes involved in maintaining carbohydrate homeostasis is a requirement to develop new therapeutic approaches for metabolic disorders.

Hepatic glucose and fructose transport and phosphorylation

Once glucose enters the hepatocytes through glucose transporter 2 (GLUT2), it is phosphorylated by glucokinase (GK or hexokinase IV) to glucose-6-phosphate (G6P). The interconversion of glucose to G6P is the major regulatory step in glucose metabolism. In fact, GK expression is positively regulated by hormones that are, in their turn, induced by insulin through the transcription factor SREBP1c. On the other hand, glucose-6-phosphatase (G6Pase) that dephosphorylates G6P, is inhibited by insulin and stimulated by glucagon (Adeva-Andany *et al.*, 2016). G6P can be converted to glycogen by glycogen synthase or further metabolized by the pentose phosphate pathway or glycolysis. During glycolysis, G6P is converted to fructose-6-phosphate that is then phosphorylated into fructose-1,6-biphosphate. The latter is cleaved by aldolase A into dihydroxyacetone-3-phosphate and glyceraldehyde-3-phosphate to form pyruvate by the end of glycolysis (Adeva-Andany *et al.*, 2016) (Figure 4).

Fructose may be transported into the cell by both GLUT2, the most expressed GLUT in hepatocytes, and GLUT5, a fructose-specific and insulin-independent transporter (Karim *et al.*, 2012, Thorens and Mueckler, 2010). It is then readily phosphorylated by fructokinase to fructose-1-phosphate and then cleaved by aldolase B to form dihydroxyacetone phosphate and glyceraldehyde. Glyceraldehyde is phosphorylated to glyceraldehyde-3-phosphate. Along with form dihydroxyacetone phosphate, these can then enter glycolysis to form pyruvate. In this way, fructose skips the regulatory mechanisms of glycolysis, thus being readily metabolized. Consequently, excessive fructose consumption results in increased lipogenesis (Mayes, 1993) (Figure 4).

Hepatic glycogen metabolism

Glucose-6-phosphate is used to synthesize glycogen in a series of reactions where glycogen synthase plays a key role. In the fed state, G6P and insulin activate glycogen synthase, promoting the increase in cellular glycogen levels. On the contrary, these molecules inhibit glycogen phosphorylase, the enzyme responsible for glycogen breakdown into glucose-1-phosphate units. In fasting, with lower circulating levels of glucose, glycogen synthesis is inhibited, and glycogen phosphorylase is activated. In cases of metabolic disorders, glycogen metabolism becomes impaired (Adeva-Andany *et al.*, 2016, Rui, 2014) (Figure 4).

Hepatic gluconeogenesis

Gluconeogenesis aims at the synthesis of glucose during long-term fasting. As glycogen becomes scarce, hepatocytes synthesize glucose from other substrates such as pyruvate, lactate and amino acids. The key regulatory enzyme of this pathway is phosphoenolpyruvate carboxylase (PEPCK), that converts oxaloacetate to phosphoenolpyruvate (Han *et al.*, 2016). PEPCK is inhibited by insulin. In metabolic disorders, despite persistent hyperinsulinemia, PEPCK activity is not inhibited due to the presence of gluconeogenic precursors, as is the case of pyruvate (Berg *et al.*, 2002) (Figure 4).

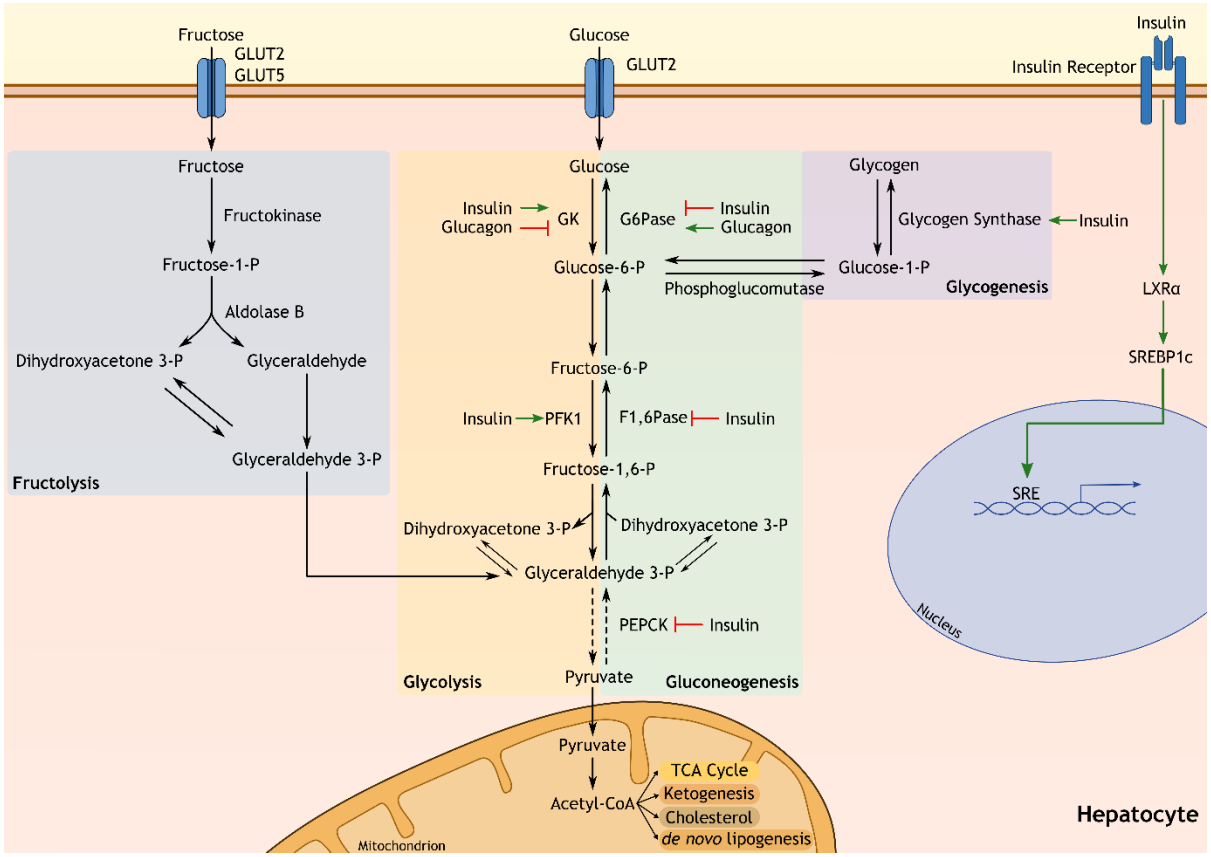

Figure 4 – Hepatic fructose and glucose metabolism. In the hepatocyte, insulin stimulates the utilization and storage of glucose as lipids and glycogen, while repressing glucose synthesis and release. Insulin stimulates the transcription of glycolytic and fatty-acid synthetic enzymes, while inhibiting the expression of gluconeogenic enzymes. These effects are dependent on a series of transcription factors and co-factors, namely SREBP1c. Insulin also regulates the enzymatic activity of glycogen synthase. In fasting, with the decrease in insulin levels and the rise in glucagon, glycolysis is inhibited, and gluconeogenesis is stimulated. On the other hand, fructose surpasses the limiting steps of glycolysis and is readily metabolized. F1,6Pase - fructose-1,6-bisphosphatase; GK - glucokinase; Glucose-6-P - glucose-6-phosphate; G6Pase - glucose-6-phosphatase; GLUT – glucose transporter; LXRα – liver X receptor α; PEPCK - phosphoenolpyruvate carboxykinase; PFK1 – phosphofructokinase 1; SRE - sterol regulatory element; SREBP1c - sterol regulatory element-binding protein 1c; TCA – tricarboxylic acid. → activation; ⊣ inhibition.

It is fundamental to unveil the metabolic pathways and its intervenients, being them either intermediates or regulators, so that appropriate diagnosis, prevention, and treatment of metabolic diseases can be successfully achieved. Besides, it is important to discover new players in these processes so that possible biomarkers of metabolic disorders may be found to improve the diagnosis.

Non-Alcoholic Fatty Liver Disease

The liver is the central organ of both lipid and glucose metabolism (Postic *et al.*, 2004). According to the American Association for the Study of Liver Diseases (AASLD), NAFLD is a broad term that includes several hepatic pathologies: non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (Loomba and Sanyal, 2013). NAFLD is the most common cause of chronic liver disease in Western countries, and has a reported prevalence of 6-35% worldwide (Bellentani, 2017). In fact, NAFLD is intimately related with IR, T2DM and obesity (Machado *et al.*, 2006). NAFLD is a consequence of several factors, namely an increased incursion of FFAs from the insulin-resistant adipose tissue, impaired processing of dietary lipids in the liver or lipid export from the hepatocytes and increased hepatic *de novo* lipogenesis (Bosserhoff and Hellerbrand, 2011).

Pathogenesis of Non-Alcoholic Fatty Liver Disease

The pathogenesis of NAFLD is not clearly understood although it is known that steatosis results from an imbalance between triglycerides production and utilization. The hepatic production of triglycerides uses FFAs derived from the diet, *de novo* lipogenesis and adipose tissue lipolysis, being the latter the major source of FFAs (Li *et al.*, 2002). NAFL, the first stage of the disease, is characterized by excessive fat accumulation in the liver with more than 5% of hepatocytes containing visible intracellular triglycerides or steatosis and affecting at least 5% of the liver total volume, in patients without other causes of steatosis (excessive alcohol consumption, hepatitis, glucocorticoids, hypothyroidism, tamoxifen, etc.; Figure 5) (Abd El-Kader and El-Den Ashmawy, 2015, Chalasani *et al.*, 2012). In approximately 25% of steatosis cases, it may progress to NASH with hepatocellular damage (Angulo, 2010, Bosserhoff and Hellerbrand, 2011). The mechanisms involved in this progression are complex and remain to be unveiled although this is thought to be linked with increased IR and FFAs release of the visceral adiposity (Stankovic *et al.*, 2014). NASH increases the probability of further progression to fibrosis, cirrhosis or even hepatocellular carcinoma, which corresponds to 20% of the individuals with NASH (Angulo, 2010, Bosserhoff and Hellerbrand, 2011).

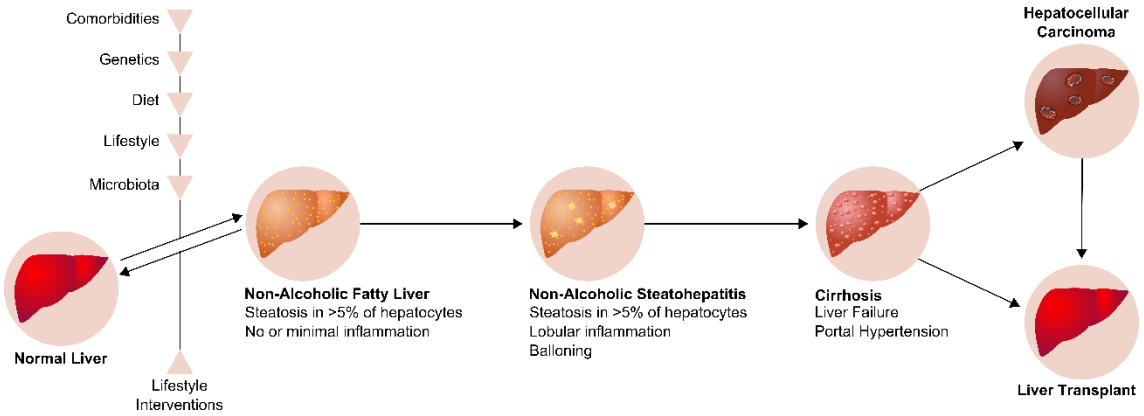

Figure 5 - Progression of non-alcoholic fatty liver disease. The liver begins to accumulate fat due to erratic lifestyle habits, genetics, microbiota leading to non-alcoholic fatty liver. It can then progress to non-alcoholic steatohepatitis (NASH) or be reverted through lifestyle interventions. NASH is characterized by lobular inflammation and ballooning. In some cases, it can progress to cirrhosis. This is a severe stage than can lead to the development of hepatocellular carcinoma (HCC), ending up in the need of liver transplant.

NAFLD was found to be complex and multifactorial, therefore several different theories have aroused to understand the underlying mechanisms for its development and progression. This led to the "two hit hypothesis", where hepatic accumulation of triglycerides due to a high fat diet, obesity and/or IR are considered to be the first hit, making the liver susceptible to the second hit, composed of further insults that sensitize this organ and lead to oxidative stress, activation of inflammatory signals and fibrogenesis (Day and James, 1998, Peverill *et al.*, 2014). However, this model was shown to be remarkably simple when compared to the complexity of the disease. Consequently, due to the presence of several factors such as lipid dysregulation, adipose tissue inflammation and adipokine impairment, inflammation, oxidative stress and IR, a "multiple hit hypothesis" was formulated to further explain the development and progression of NAFLD (Buzzetti *et al.*, 2016, Polyzos *et al.*, 2012). This hypothesis states that dietary factors, that may be or may not be associated with obesity, lead to gut microbiome dysfunction, increased levels of both cholesterol and FFAs, as well as adipocyte proliferation and the development of IR (Tilg and Moschen, 2010). The latter will have consequences on the adipose tissue and the liver. In fact, IR aggravates adipocyte dysfunction, inducing lipolysis and the release of proinflammatory cytokines (Flock *et al.*, 2011). On a hepatic level, some authors propose that IR is the main cause of NAFLD. In fact, mice without hepatic InsR expression develop IR, severe glucose intolerance, and present hyperinsulinemia, due to increased insulin secretion but decreased insulin clearance, suggesting that hepatic insulin signaling is a key player in controlling peripheral insulin responsiveness (Michael *et al.*, 2000). Moreover, IR increases *de novo* lipogenesis. The increased levels of FFAs lead then to hepatic inflammation, which results from the mitochondrial dysfunction and oxidative stress due to excessive levels of cholesterol, FFAs and lipid metabolites. Moreover, intestine permeability might be observed, increasing the circulating levels of molecules that promote the release of proinflammatory cytokines (Buzzetti *et al.*, 2016).

Management of NAFLD

The exponential increase in the prevalence of obesity and NAFLD is mostly due to lifestyle changes, namely a higher consumption of high energy/readymade food and the decrease of physical activity (Fan *et al.*, 2017). Thus, lifestyle modification through caloric restriction and increased physical activity is the first-line intervention for NAFLD. The resulting weight loss leads to a reduction in IR and amelioration of liver enzymes levels, while the physical activity amends the progression of NAFLD (Scaglioni *et al.*, 2013, Suzuki *et al.*, 2005). In fact, it was shown that even in the absence of weight loss, exercise has beneficial effects on intrahepatic triglycerides (Katsagoni *et al.*, 2017) and IR (Oh *et al.*, 2014). Currently, there are no Food and Drug Administration (FDA) nor European Medicines Agency (EMA)-approved therapeutic agents for NAFLD. However, as the awareness for NAFLD grows, so does the interest in developing new therapeutic agents that could be used to treat NAFLD. Several compounds that target different stages and molecular events of the disease are being developed and some already commercially available drugs for other diseases have been restudied for the treatment of NAFLD. However, it has to be taken into account that due to metabolic alterations, obesity and NAFLD increase the risk of drug-induced liver injury (Fromenty, 2013). These impairments may be related with cytochrome P450, IR or even lipogenic transcription factors (Aubert *et al.*, 2011).

3. Paraoxonase-1 and Apolipoprotein J: The search for new biomarkers of metabolic disorders – focus on dyslipidemia

Histologically, NAFL encompasses any degree of steatosis with or without lobular inflammation but where ballooning is absent. On the other hand, NASH is characterized by the presence of ballooning, a form of hepatocyte cell death where the cells increase in size. However, it is important to distinguish between NAFL and NASH, as the first has a better prognosis than the latter (Dyson *et al.*, 2014). Although some clues on liver disease may appear through the analysis of liver enzymes in a blood test, the best way to diagnose steatosis is either by liver biopsy or by imaging techniques. The latter include ultrasound and proton magnetic resonance spectroscopy. These techniques do not, however, exclude NASH, as they purely quantify steatosis (Li *et al.*, 2018, Yki-Järvinen, 2016). At this point, the only reliable way to diagnose NASH is by liver biopsy, then applying standardized pathologic staging systems (Li *et al.*, 2018). However, this has numerous limitations, namely invasiveness, low patient acceptance rates, and low representativity of the tissue. In the last few years, Fibroscan® emerged as a new alternative and has been used in early clinical studies (Celerion, 2019). Its technology is based on transient elastography, being able to measure both liver stiffness and steatosis. Fibrosis is then calculated as a function of liver stiffness, and the reduction of the signal is proportional to steatosis (Patel and Wilder, 2014). However, as NAFL is a silent disease, the need for biomarkers that can be easily detected/measured is of the uttermost importance. In fact, in the last few years, the search for biomarkers to diagnose NAFLD and its progression has become a high priority for clinical routine. Biomarkers offer a potential diagnostic and/or prognostic tool for disease onset and progression. Specifically, an ideal biomarker should be predictive of the onset of the disease; it would give insight into the biological processes that result in the onset or progression of NAFLD; and it might also be a surrogate biomarker of the underlying disease process, therefore making it useful for monitoring a patient's response to an intervention (Sahebkar *et al.*, 2018). Unfortunately, to date, many critical gaps exist in the reference interval database of most of the biomarkers that have been identified for the evaluation of hepatic steatosis. Given that most metabolic disorders are characterized by a pro-oxidant environment, endogenous antioxidant proteins as paraoxonase-1 could be possible biomarkers.

Paraoxonase-1

The paraoxonase (PON) enzyme family is constituted by three members: PON1, PON2 and PON3. Human PONs are encoded by three adjacent but distinct genes located on chromosome 7 (Primo-Parmo *et al.*, 1996). In mammals, these genes have 81–95% homology and the resultant proteins share 79–95% of their sequence at the amino acid level (Rajkovic *et al.*, 2011). Based on the structural homology and from an evolutionary point of view, paraoxonase 2 (PON2) is the oldest member of this family, being paraoxonase 1 (PON1) and paraoxonase 3 (PON3) probably a result of gene duplication (Primo-Parmo *et al.*, 1996). Despite that, the family was named due to early studies on PON1 ability to hydrolyze paraoxon, the toxic metabolite of the insecticide parathion (Dias *et al.*, 2014, Furlong

et al., 2016). On the contrary, PON2 and PON3 do not have the ability to degrade xenobiotics (Reddy *et al.*, 2001). Besides paraoxonase activity, PON1 has lactonase and ester hydrolase activities, being able to hydrolyze thiolactones, unsaturated aliphatic esters, aromatic carboxylic esters and carbamates (Davies *et al.*, 1996). All the members of this family degrade lipid peroxides in high-density lipoproteins (HDL) and low-density lipoproteins (LDL) (Reddy *et al.*, 2001). In humans, PON1 and PON3 are mainly expressed in the liver being secreted into the bloodstream, where the enzymes are mainly found bound to HDL (Mackness and Mackness, 2015). Besides the liver, PON3 is also expressed in the kidney (Ferretti and Bacchetti, 2012). In contrast, PON2 is expressed in several organs such as liver, lungs, placenta, testis and heart but it is not found in circulation (Ng *et al.*, 2001). Within the cell, PON2 is located in the nuclear envelope, endoplasmic reticulum, and in the inner mitochondrial membrane (Ng *et al.*, 2001). Here, PON2 is found associated with respiratory complex III being essential for the correct function of the electron transport chain (Devarajan *et al.*, 2011).

PON1 (EC 3.1.8.1), which is the most studied member of the PON family, is classified as an aryldialkylphosphatase (Furlong *et al.*, 2016). It was first described in the 1940's due to its ability to hydrolyze organophosphate compounds (Mazur, 1946). The gene that encodes for PON1 is located on chromosome 7 (7q21.3-q22.1) in humans and in the proximal region of chromosome 6 in mice (Humbert *et al.*, 1993, Sorenson *et al.*, 1995). The coding region of the gene has nine exons and eight introns, which contain many polymorphic CA repeats. Remarkably, it appears that the 5'-unstranslated region (UTR) of the PON1 gene does not have a TATA box (Primo-Parmo *et al.*, 1996). PON1 transcription is highly regulated by specificity protein 1 (Sp1), protein kinase C (Arii *et al.*, 2010, Tan and Khachigian, 2009), and peroxisome proliferator-activated receptors (PPARs) (Moraes *et al.*, 2006). Sp1, whose binding site is present in the *PON1* gene, represents a positive regulator of PON1 (Brophy *et al.*, 2001). It was demonstrated in hepatic cell lines that an increase of Sp1 enhances PON1 transcription; on the other hand, its absence leads to decreased gene transcription (Osaki *et al.*, 2004). Moreover, protein kinase C, especially the zeta isoform, increases the binding intensity of Sp1 to PON1 DNA promoter, thus having a role in transcription (Arii *et al.*, 2010, Osaki *et al.*, 2004).

More than 200 single nucleotide polymorphisms (SNPs) were already found on *PON1* gene, contributing to interindividual differences in PON1 concentration and activity (Richter *et al.*, 2010). Of those, there are two common polymorphisms in the coding region of the *PON1* gene with different biological consequences. The leucine to methionine substitution at the position 55 (L55M) affects PON1 concentration: *PON1L* allele presents more PON1 mRNA than *PON1M* allele, leading to an increase in PON1 levels (Garin *et al.*, 1997, Leviev *et al.*, 1997). On the other hand, the glutamine to arginine substitution at the position 192 (Q192R) correlates with PON1 activity as it leads to differences in their catalytic activity towards synthetic substrates. In this case, the *PON1R* allele has higher paraoxonase and arylesterase activity than *PON1Q* allele and the latter shows higher lactonase activity than *PON1R* (Adkins *et al.*, 1993, Rainwater *et al.*, 2009). Moreover, several other polymorphisms have been identified in the non-coding region of the *PON1* gene. One of the most important is the cytosine to thymidine substitution at position 107 (C107T), where the *PON1C* allele leads to higher

serum levels of PON1 (James *et al.*, 2000). Besides genetic factors, PON1 activity and serum levels are also modulated by diet, age, lifestyle, pharmaceutical interventions and epigenetic factors (Costa *et al.*, 2005, Schrader and Rimbach, 2011). Altogether, these factors contribute to the observed interindividual variability of PON1 (Draganov and La Du, 2004).

PON1 is a 43 kDa glycoprotein composed of 354 amino acids, and its structure is a six-bladed ß-propeller, where each blade consists of four ß-sheets (She *et al.*, 2012). Moreover, it has two calcium binding sites that are essential for PON1 activity. One is located at the top of the structure and is thought to be related with the catalytic activity and the other is in the central section and may contribute to the structural stability of the protein (Harel *et al.*, 2004, Harel *et al.*, 2007). In fact, calcium removal with chelating agents irreversibly destroys PON1's activity and stability (Kuo and La Du, 1998).

When secreted into the bloodstream, PON1 is found mainly associated with HDL (Moya and Manez, 2018), as HDL guarantees shelter for the hydrophobic N-terminal region of PON1 in aqueous environment (Sorenson *et al.*, 1999). This stabilizes PON1 and creates a hydrophobic environment that optimizes its function (Deakin *et al.*, 2002a). Besides facilitating its secretion from the liver, HDL may also be essential for the interaction of PON1 with its substrates (Sorenson *et al.*, 1999). One of the questions that remains to be addressed concerns the specificity with which PON1 associates to lipoproteins. In fact, PON1 does not bind to LDL, which is a major plasmatic lipoprotein. However, PON1 is found within chylomicrons (Fuhrman *et al.*, 2005) and VLDL, the other principal lipoprotein subfraction found in plasma, although to a lesser extent comparing to HDL (James and Deakin, 2004). PON1-HDL is also commonly associated with two apolipoproteins, apolipoprotein A-I (apoA-I) and apolipoprotein J (ApoJ) (Park *et al.*, 2014). The former is a major protein component of HDL, that is known to improve the stability of PON1-HDL interaction (Sorenson *et al.*, 1999).

Although PON1 was first discovered due to its paraoxonase activity, its substrates are man-made and have no physiological relevance. Thus, PON1 biological activity can be divided into three categories: lactonase, esterase, and phosphotriesterase, consequently responsible for the catalysis of different substrates (Draganov *et al.*, 2005). Besides degrading organophosphates such as paraoxon, arylesters, cyclic carbonates, glucuronides, and estrogen esters (Rajkovic *et al.*, 2011, Sorenson *et al.*, 1999), this detoxifier protein also hydrolyses a large diversity of lactones, as well as pharmaceutical agents, namely statins (Khersonsky and Tawfik, 2005). The latter are used in the treatment of CVD and exert its effects through the inhibition of cholesterol synthesis in cells and decrease of LDL (Pahan, 2006). As PON1 also degrades oxidized phospholipids and therefore playing a role in the antioxidant system, alterations in circulating PON1 levels and activity have been associated with a variety of diseases involving oxidative stress. In fact, the interest on this protein has increased due to evidences that decreased PON1 activity is related to several non-communicable diseases such as cancer, obesity, DM, CVD, neurodegenerative diseases and NAFLD (Aslan *et al.*, 2011, Fedelesova *et al.*, 2017).

PON1 and lipid metabolism

It has been demonstrated that the absence of PON1 in C57BL/6J mice results in a decrease of glycerol, polyunsaturated fatty acids (PUFA)/monounsaturated fatty acids (MUFA) ratio and 3-hydroxybutyrate (Garcia-Heredia *et al.*, 2013). Lower levels of these lipid metabolism intervenient, in comparison with normal conditions, suggest a decrease in lipolysis and decreased fatty acid oxidation, that can be attributable to PON1 impairment. The levels of carnitine were also decreased, suggesting depressed fatty acid oxidation. In fact, carnitine is necessary for the transport of long-chain fatty acids into the mitochondria. Moreover, levels of several lysolipids were reduced by PON1 deficiency, pointing towards reduced membrane remodeling and/or breakdown (Garcia-Heredia *et al.*, 2013). There is also a reduction in bile acid metabolism, with a decrease in squalene levels, that is essential for the absorption of lipids from diet and hydrophobic vitamins, such as A, D, E, K (Garcia-Heredia *et al.*, 2013). As lipids affect PON1 levels and activity, the drugs that impact on lipid levels are also expected to affect PON1. In fact, it has already been observed that statins, one of the most important classes of lipid lowering drugs, may preserve PON1 activity due to its antioxidant properties (Aviram *et al.*, 1998a). Moreover, simvastatin, a drug from the same class, was found to impact on PON1 levels, through sterol regulatory element-binding protein 2 (SREBP2). SREBP2 is known to bind to the PON1 promoter, leading to its upregulation. This is apparently made in an interactive manner with Sp1 (Deakin *et al.*, 2003).

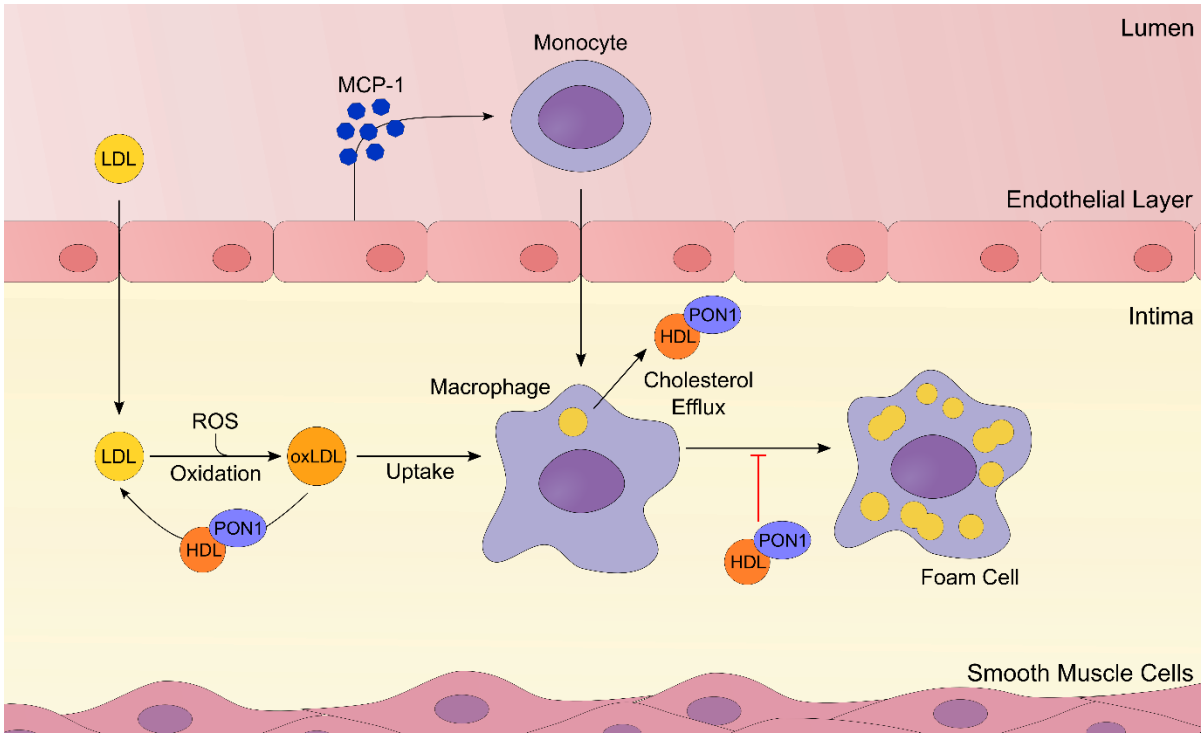

Figure 6 - The role of paraoxonase-1 (PON1) in the pathophysiology of atherosclerosis. Inflammation and oxidative stress lead to the oxidation of low-density lipoproteins (LDL). The release of monocyte chemoattractant protein-1 (MCP-1) from endothelial cells will lead to the recruitment of monocytes. These will differentiate into macrophages, that will internalize the oxidized LDL (oxLDL), becoming foam cells. PON1 hydrolyzes the oxLDL, reverting it to LDL, and promotes cholesterol efflux from macrophages, inhibiting the progression of atherosclerosis. ⊣ inhibition

PON1 was also found to inhibit macrophage cholesterol biosynthesis (Rozenberg *et al.*, 2003b). In fact, mouse peritoneal macrophages from PON1 knockout mice had increased cellular cholesterol biosynthesis and PON1 administration led to a direct inhibition of this process. This mechanism is probably related to PON1 phospholipase-A2-like activity, which results in lysophosphatidylcholine formation and inhibition of cellular cholesterol biosynthesis (Rozenberg *et al.*, 2003b). Moreover, PON1 stimulates HDL-mediated cholesterol efflux from macrophages and attenuates oxidized-LDL uptake by macrophages (Tavori *et al.*, 2011, Tavori *et al.*, 2009) (Figure 6).

PON1 and glucose homeostasis

PON1 is involved in the regulation of glucose metabolism, as it influences fasting blood glucose levels, glucose tolerance and insulin sensitivity (Koren-Gluzer *et al.*, 2013). It has been demonstrated that PON1 upregulates GLUT4 expression in the muscle in an insulin receptor-independent manner (Koren-Gluzer *et al.*, 2013). In fact, PON1 can regulate the expression and translocation of GLUT4 via inhibition of P38 mitogen-activated protein kinases (p38MAPK) activity, leading to reduced insulin receptor substrate-1 (IRS1) serine phosphorylation and to enhanced IRS1 tyrosine phosphorylation. Moreover, it was demonstrated that PON1 SH group and PON1 lactonase activity at catalytic site His115 were key players in the increase in GLUT4 expression (Khersonsky and Tawfik, 2006, Koren-Gluzer *et al.*, 2013). Besides regulating glucose uptake, PON1 directly regulates some players of glycolysis, namely 3-phosphoglycerate, phosphoenolpyruvate and lactate. On the other hand, PON1 negatively affects the levels of fructose-1,6-biphosphate in glycolysis, as well as levels of components of the pentose phosphate pathway, namely ribulose 5-phosphate, ribose 5-phosphate and xylonate (Garcia-Heredia *et al.*, 2013). Accordingly, in the absence of PON1, the activity of glycolysis and the Krebs cycle is decreased and hence the energy obtained in these two pathways is impaired. In contrast, pentose phosphate pathway becomes more activated, which implies the increase of the NADPH generated (Garcia-Heredia *et al.*, 2013).

PON1 and diet

Diet composition has a significant role in PON1 modulation. A lipid enriched diet is closely associated with inflammation and oxidative stress (De Keyzer *et al.*, 2009, Kris-Etherton *et al.*, 1997). In fact, the increase of dietary lipids results in increased production of ROS, lipid hydroperoxides and inflammatory cytokines, such as TNFα, interleukin-1 and IL-6 (Ferretti and Bacchetti, 2012). These factors contribute to a decrease in PON1 expression and consequently decreased hepatic PON1 secretion. This reduction of *PON1* mRNA expression and HDL synthesis are direct consequences of decreased PPARδ, induced by elevated levels of free radicals and lipid peroxidation products in liver (Ferretti and Bacchetti, 2012, Marsillach *et al.*, 2009). Thus, serum HDL-PON1 activity appears to be diminished in high-fat diet, leading to the impaired reverse cholesterol transport (Bonen *et al.*, 2006).

High-sucrose diet influences glucose metabolism, as sucrose hydrolysis into glucose and fructose increases its plasmatic levels (Diniz *et al.*, 2006). On the other hand, as pentose and hexose molecules are converted in triglycerides, high-sucrose diet leads to the increase of circulating VLDL, due to

hypertriglyceridemia (Flatt, 1970, Kannan *et al.*, 1981). It has been suggested that VLDL may promote the secretion of antioxidant enzymes by the liver (Deakin *et al.*, 2005). Several *in vivo* studies have demonstrated that high-sucrose diet, as soon as two weeks after the beginning of the diet, induces both hyperlipidemia and oxidative stress, which are known to decrease PON1 activity *per se* (Busserolles *et al.*, 2002, Diniz *et al.*, 2006). However, a study that directly evaluated PON1 activity in rats after three to five weeks of high-sucrose feeding observed a significant increase in PON1 activity (Macan *et al.*, 2010).

Mediterranean diet is known as one of the best diets protecting against CVD. It was already demonstrated that the consumption of a Mediterranean diet increases postprandial PON1 activity (Blum *et al.*, 2006). These results were similar to those obtained after the administration of omega-3 PUFA to individuals with familial combined hyperlipidemia (Calabresi *et al.*, 2004). The segmentation of the components of the Mediterranean diet showed that these effects may be attributable to the consumption of extra virgin olive oil (Loued *et al.*, 2013). Moreover, some components of the olive oil, not only improve PON1 activity, but also its hepatic mRNA expression and protein levels (Lou-Bonafonte *et al.*, 2015). Identifying and characterizing dietary components that favor PON1 activity and/or expression may lead to the development of products to enhance the protective role of PON1.

PON1 and Disease

PON1 and Atherosclerosis

Atherosclerosis is a lipid-driven chronic inflammatory disease caused by the combination of lipid accumulation and immune cells within the atherosclerotic plaque (Moore *et al.*, 2013). A key early step in the development of atherosclerosis is the oxidation of serum LDL. The oxidized products are scavenged by macrophages which may transform into foam cells (Figure 1). These will then become fatty streaks in the endothelium and form atheromatous plaques (La Du *et al.*, 1999). Studies on animal models have demonstrated that overexpression of PON1 leads to an increased resistance to inflammation and atherosclerosis (Tward *et al.*, 2002) whereas PON1 knockout mice are more susceptible to lipoprotein oxidation and increased inflammation (Shih *et al.*, 2000). Moreover, macrophages of PON1 knockout mice present decreased glutathione concentrations and increased oxidative stress markers (Rozenberg *et al.*, 2003a), resulting in increased oxLDL and foam cell formation (Aviram and Rosenblat, 2004). One of the first events of plaque formation is the upregulation of MCP-1 secretion by oxLDL. PON1 inhibits the secretion of MCP-1 in several ways. In one hand, inhibits its secretion from endothelial cells. On the other hand, prevents LDL-derived oxidized phospholipid formation, that are known to stimulate adhesion of monocytes to the endothelial cells and MCP-1 production (Mackness *et al.*, 2004). Moreover, the formation of lysophosphatidylcholine induced by the HDL-associated PON1 hydrolytic action on macrophage phospholipids enhances HDL binding to macrophages and consequently enhances cholesterol efflux (Rosenblat *et al.*, 2005) (Figure 6).

Oxidized lipoproteins, such as LDL and HDL, may be converted into inflammatory signals due to their oxidation by ROS (Navab *et al.*, 2006). PON1, isolated or in combination with HDL, protects against LDL oxidation promoted by cooper ion and free radicals, including ROS (Watson *et al.*, 1995). This is mainly due to its ability to hydrolyze peroxide phospholipids in the structure of oxidized LDL and to neutralize the effects of atherogenic lipid peroxides (Aviram *et al.*, 1999, Mackness *et al.*, 1997, Watson *et al.*, 1995). The main lipid peroxides which are subjected to hydrolysis by PON1 are triglyceride hydroperoxides, truncated oxidized FA from phospholipid and cholesteryl ester (Aviram *et al.*, 1998b). Lipid hydroperoxides have a great inflammatory component and cause alterations in carbohydrate and lipid metabolism, particularly in the metabolism of hexoses, glycolysis, Krebs cycle and phospholipid metabolism decreasing phospholipid synthesis and increasing its degradation (Garcia-Heredia *et al.*, 2013). Furthermore, it has been suggested that PON1 is inactivated by oxLDL, due to an interaction of the enzyme free sulfhydryl group (cysteine-284) and oxidized lipids formed during LDL oxidation, such as oxidized phospholipids, oxidized cholesteryl ester or lysophosphatidylcholine (Aviram *et al.*, 1999). Therefore, oxLDL is responsible for inactivating PON1 and consequently the arylesterase activity of PON1 decreases, as well as the ability of PON1 to protect against LDL oxidation (Aviram *et al.*, 1999). Undeniably, metabolic disorders with a prooxidant component lead to the increase of ROS and hence result in increased oxidative stress and decreased PON1 antioxidant activity and bioavailability. Moreover, as PON1 inhibits the oxidation of LDL, it prevents the upregulation of proinflammatory cytokines and chemokines, such as MCP-1 produced by cells in the vessel wall (Watson *et al.*, 1995). Consequently, PON1 reduces the proatherogenic properties of LDL (Mackness and Mackness, 2010), thus indicating its role as a protector of cell membrane integrity and cardiovascular complications.

Regarding HDL, PON1 stimulates the antiatherogenic effects of HDL in reverse cholesterol transport, by inducing the mobilization of cholesterol within macrophages and promoting its efflux to HDL (Figure 6) (Aviram *et al.*, 1998b). However, the decrease of PON1 activity and chronic inflammation leads to changes in the HDL proteome ultimately promoting this lipoprotein's dysfunction, as well as a decrease of its anti-oxidant and anti-inflammatory capacity (Farbstein and Levy, 2012). Therefore, cholesterol efflux and reverse cholesterol transport become impaired. As the ability of HDL to modulate oxLDL and to protect it from further oxidation decreases, plasma levels of LDL increase. Consequently, the pro-oxidant status also increases. The increase in circulating oxLDL causes a rise of MCP-1, as well as activation of the endothelial cell (Clay *et al.*, 2001, Mackness *et al.*, 2004) promoting atherosclerosis development. This set of factors result in increased risk to develop CVD and associated complications (Barter and Rye, 1996, Taskinen, 2003).

PON1 and Diabetes *mellitus*

Most studies have found that PON1 activity is decreased in both T1DM and T2DM (Boemi *et al.*, 2001, Craciun *et al.*, 2016, Letellier *et al.*, 2002). Although this decrease is probably independent of PON1 polymorphisms, PON155L has been associated with diabetic retinopathy (Mackness *et al.*, 2000). Moreover, in healthy individuals, the PON1L55M polymorphism has been linked to impaired β-cell function, impaired glucose disposal, and increased IR (Chiu *et al.*, 2004, Deakin *et al.*, 2002b). The

mechanism by which PON1 is reduced in DM remains poorly understood but might be linked to blood glucose levels. Under hyperglycemic conditions both PON1 expression and activity are affected. High concentrations of hexose molecules are accompanied by IR and increased lipid accumulation, particularly of diacylglycerol (Nakajima *et al.*, 2000). This causes activation of numerous protein kinase C isoforms (Laybutt *et al.*, 1999), which are responsible for the phosphorylation of Sp1. As Sp1 is a positive regulator of *PON1*, its phosphorylation will enhance *PON1* transcription (Osaki *et al.*, 2004). In metabolic disorders, the presence of several factors linked to abnormally high glucose levels such as hyperinsulinemia, dyslipidemia and oxidative stress, leads to a decrease of PON1 activity, especially due to glycation of HDL-PON1 (Mastorikou *et al.*, 2008). In fact, in order to compensate this decrease in PON1 activity, the mRNA expression and synthesis of serum PON1 are both increased (Ikeda *et al.*, 2008). It has also been demonstrated that levels of PON1 are significantly decreased in IR (Gateva *et al.*, 2016). Therefore, in both types of DM, serum PON1 activity and concentration are significantly decreased, since hyperglycemia, oxidative stress and IR coexist (Deakin and James, 2004).

Glycation or non-enzymatic glycosylation of PON1 is another consequence of hyperglycemia associated with DM (Yu *et al.*, 2017). This process leads to the inhibition of the paraoxonase activity of PON1. Glycation is also responsible for HDL dysfunction and catabolism (Witztum *et al.*, 1982), so glycated HDL is unable to metabolize membrane lipid peroxides and to maintain the cholesterol efflux (Mastorikou *et al.*, 2008). It was found that HDL isolated from individuals with lower PON1 activity are more susceptible to lipid peroxidation in hyperglycemic conditions than HDL from subjects with higher PON1 activity (Ferretti *et al.*, 2001). It has been demonstrated that glycated PON1 also results in endothelial dysfunction in patients with T2DM through endoplasmic reticulum stress which is widely associated with other pathologies such as atherosclerosis, obesity, NAFLD, neurodegenerative disorders and cancer (Ozcan and Tabas, 2012). This process is triggered by hyperglycemia and oxidative stress (Yu *et al.*, 2017). Therefore, in metabolic disorders, the activity of PON1 and anti-inflammatory and anti-atherosclerotic properties of serum HDL-PON1 complex are significantly decreased due to inactive oxidation and glycation of both free PON1 and HDL-PON1.

PON1 and Obesity

PON1 activity was reported to be significantly lower in obese individuals when compared to controls (Cervellati *et al.*, 2018, Ferretti *et al.*, 2005). In fact, this decrease was accompanied by an increase in the levels of lipid hydroperoxides in HDL and LDL from obese subjects, suggesting that obese individuals are more susceptible to oxidative damage (Ferretti *et al.*, 2005). Moreover, BMI was already found to be an independent predictor of PON1 activity (Bajnok *et al.*, 2008). This decrease in PON1 activity in obese subjects is independent of the age, as it was also found in young subjects (Koncsos *et al.*, 2010). Moreover, PON1 arylesterase activity was negatively correlated with leptin concentrations but positively correlated with adipokine levels. As was the case for IR the study of the molecular mechanisms promoting fat accumulation is also fundamental. Again, PON1, with its antiatherogenic properties may be an ideal candidate in the pathophysiology of obesity.

PON1 and Non-Alcoholic Fatty Liver Disease

As PON1 has a protective effect against oxidative stress, it is expected to have a role in NAFLD. In PON1 knockout mice, a high-fat/high-cholesterol diet caused increased oxidative stress and metabolic alterations that led to the development of steatosis (Garcia-Heredia *et al.*, 2013). Moreover, these mice presented decreased glycolysis and Krebs cycle, as well as the urea cycle. On the contrary, triglyceride and phospholipid synthesis were significantly increased (Garcia-Heredia *et al.*, 2013). A more recent study has demonstrated that serum PON1 activity is paradoxically maintained in patients with fatty liver index (FLI) above 60 despite low HDL (van den Berg *et al.*, 2019). Moreover, a study using paraoxon as substrate, demonstrated that PON1 activity was decreased in patients with hepatic steatosis, compared with healthy subjects (Atamer *et al.*, 2008).

Apolipoprotein J

Clusterin (CLU) is a ubiquitously expressed glycoprotein coded by a single copy gene on chromosome 8. It was originally found in the ram *rete testis* and was named due to its ability to cluster Sertoli cells (Fritz *et al.*, 1983). However, it was later found in other tissues and body fluids and given other names based on its location and function, such as androgen repressed protein, sulfated glycoprotein-2, complement lysis inhibitor and ApoJ. Only in 1992, due to the sequencing of their cDNA and protein sequence, researchers agreed that all of these proteins were, in fact, CLU (Fritz and Murphy, 1993). From then, it is widely accepted that CLU is expressed in several tissues and body fluids (De Silva *et al.*, 1990). As a consequence, CLU is linked to numerous biological processes such as sperm maturation, lipid transport, membrane remodeling, inhibition of the complement system, chaperone function, and regulation of cell survival and cell death pathways (Foster *et al.*, 2019, Park *et al.*, 2014, Wong *et al.*, 1994).

CLU has two isoforms: a nuclear isoform (nCLU), that has proapoptotic functions (Yang *et al.*, 2000); and a secretory form (sCLU) that has antiapoptotic features and is also named as ApoJ (Yao *et al.*, 2018). nCLU is the less studied molecular form, although it is known that when it is present in the nucleus above what is found in the cytoplasm, the cell is about to enter apoptosis (Bettuzzi and Rizzi, 2009). In its secretory form, ApoJ is known to bind to an extensive network of metabolic mediators, as is the case of proinflammatory cytokines, leptin, and growth hormone (Falgarone and Chiocchia, 2009). Importantly, it is found in the lipid-poor subclass of HDL-c, which also contains ApoAI and PON1 (Jordan-Starck *et al.*, 1992).

ApoJ has chaperone functions due to a heat shock element-like motif in the *CLU* promotor, being the first known chaperone to act outside of the cell (Carver *et al.*, 2003, Michel *et al.*, 1997). Thus, besides preventing the aggregation of denatured proteins, ApoJ facilitates the uptake of these proteins into surrounding cells to allow their removal through lysosomal digestion (Bartl *et al.*, 2001). ApoJ has been linked to numerous diseases, namely atherosclerosis, cancer and Alzheimer's disease (Foster *et al.*, 2019). ApoJ was already reported to be increased in individuals with T2DM and obesity (Trougakos *et al.*, 2002), and specifically, in individuals with IR (Seo *et al.*, 2018). Moreover, it was found that ApoJ levels were positively correlated with the degree of IR (Seo *et al.*, 2018). Regarding

the liver, increased levels of ApoJ in the serum of patients with primary hepatocellular carcinoma are associated with poor prognosis (Yao *et al.*, 2018). Furthermore, ApoJ was shown to be a better predictor than α-fetoprotein, a marker for primary hepatocellular carcinoma, showing that ApoJ may be used as a prognosis biomarker for liver disease (Nafee *et al.*, 2012).

4. Impact of Dysmetabolism on the Male Reproductive System

Concurrent with the drastic increase in the prevalence of metabolic disorders such as obesity and diabetes, we have also been witnessing a dramatic decline in fertility rates. In fact, semen quality has declined in European populations from a few decades ago until now (Slama *et al.*, 2004, Van Waeleghem *et al.*, 1996). Furthermore, these two trends appear to be interrelated for several reasons. One of the possible explanations is the enlarged frequency of men on reproductive age suffering from metabolic disorders (Delfino *et al.*, 2007). In fact, the metabolism of testicular cells is crucial for the normal occurrence of spermatogenesis. Besides, glucose, insulin and lipids have a key role in the control of the metabolic cooperation established between Sertoli cells - the somatic cells of the testis - and developing germ cells (Alves *et al.*, 2012, Rato *et al.*, 2013). Thus, the dysregulation promoted by either obesity or DM in the testicular metabolic cooperation might be a key factor to the decrease in fertility rates observed in countries with high incidence of metabolic diseases. Nowadays, there is evidence from several studies performed either *in vitro* or *in vivo*, that the presence of metabolic disorders or some of their main features have a profound impact on the reproductive potential of males (Alves *et al.*, 2012, Rato *et al.*, 2013). Moreover, in the last years, several studies have been performed to unveil the mechanisms by which metabolic diseases alter the reproductive potential of males.

Oxidative Stress and Its Effects on Male Reproduction

Concerning male reproductive potential, ROS play a crucial role in sperm function as second messengers, namely in capacitation, acrosome reaction (de Lamirande and O'Flaherty, 2008), and sperm-oocyte fusion (de Lamirande and Gagnon, 1993). However, when the antioxidant capacity of the cells is insufficient to neutralize ROS, either by an increase of ROS or by a decrease in antioxidant defenses, oxidative stress occurs (Aitken and Clarkson, 1987). Oxidative stress may then impair sperm cells through damages in plasma membrane and deoxyribonucleic acid (DNA) integrity affecting sperm motility and viability, sperm-oocyte fusion, development of embryo and even maintenance of pregnancy (Bromfield *et al.*, 2015). One of the main reasons for spermatozoa being so susceptible to oxidative stress is the high percentage of PUFA that they contain (Lenzi *et al.*, 1994). The attack to the latter by free radicals leads to the formation of lipid radicals. These combine with oxygen, the universal electron receptor, generating a lipid peroxyl radical (Ayala *et al.*, 2014). Then, lipid radicals are formed because lipid peroxyl radical extracts hydrogen atoms from adjacent lipids in order to stabilize as a hydroperoxide (Moazamian *et al.*, 2015). In consequence of lipid peroxidation, lipid aldehydes (such as 4-hydroxynonenal (4-HNE)) are formed and bind to mitochondrial proteins, stimulating the generation of even more free radicals (Ayala *et al.*, 2014). This further enhances lipid peroxidation in a vicious cycle that leads spermatozoa to apoptosis. However, and due to the structure of spermatozoa, the nucleases activated in the midpiece, either in the cytoplasm or in mitochondria, cannot enter the nuclear compartment. Hydrogen peroxide is the only product of apoptosis that can pass from the midpiece to the sperm head and consequently damage the DNA. Thus, this is the main

reason why the great percentage of DNA damage in spermatozoa has oxidative origin (Aitken *et al.*, 2015).

Implications of metabolic diseases on sperm quality

As previously described, there is a growing percentage of couples with fertility problems. It has been shown that many aspects related to lifestyle may be implicated in their lower reproductive potential. Some metabolic disorders are intimately linked with wrong dietary habits, which already have been shown to have negative effects on male reproductive function, namely on sperm quality.

Although only recently attention has been given to the effects of obesity on male reproductive system, it is now known that obesity may harmfully affect male reproductive function due to thermal, endocrine, and genetic mechanisms (Practice Committee of the American Society for Reproductive Medicine, 2015). In fact, obese men have compromised testicular thermoregulation due to a sedentary lifestyle and to increased fat deposition in the abdominal, suprapubic, spermatic cord and upper thigh areas (Durairajanayagam *et al.*, 2015). Although it is known for several years that this leads to suppressed spermatogenesis, the mechanisms are not yet fully clarified (Durairajanayagam *et al.*, 2015). However, it is possible that the suppressed spermatogenesis may be also due to increased oxidative stress, leading to the apoptosis of developing germ cells (Shiraishi *et al.*, 2010).

Additionally, adipose tissue is now known as an important organ of hormone production and metabolism, thus accumulation of increasing quantities of body fat may interfere with the hormonal regulation of testicular function. Numerous studies observed that metabolic parameters linked with obesity, namely high levels of plasma cholesterol and triglycerides, impair testicular function, leading to decreased semen quality and thus infertility (Jones *et al.*, 1979). Moreover, due to a higher percentage of body fat, obese men have reduced levels of total and bioavailable testosterone, as well as decreased inhibin B, that are accompanied by a decrease in luteinizing hormone pulse amplitude (Vermeulen *et al.*, 1993). These hormonal changes are suggested to be caused by an estrogen negative-feedback inhibition, due to increased adipose-derived aromatase activity (Roth *et al.*, 2008, Wake *et al.*, 2007), along with decreased formation of inactive 2-hydroxyestrogens (Palmer *et al.*, 2012, Wake *et al.*, 2007). Therefore, Leydig cells in obese men secrete less testosterone, and these levels are negatively correlated with leptin (Isidori *et al.*, 1999) and fasting insulin levels (Hofny *et al.*, 2010). Along with this, sex hormone-binding globulin (SHBG) has also a preponderant role in obesity-related male infertility. SHBG is produced in the liver and then secreted into the blood, where it binds androgens and estrogens, regulating their bioavailability. However, SHBG production and secretion is under the control of hormones and nutritional factors in the liver (Pugeat *et al.*, 2010). In fact, both *in vitro* and *in vitro* studies suggest that insulin lowers SHBG production (Kalme *et al.*, 2003, Peiris *et al.*, 1993). These findings indicate that IR, directly or indirectly through hyperinsulinemia, may have a role in the regulation of SHBG production. BMI is considered a major determinant of SHBG plasma concentrations and it is known that obese individuals have low levels of this globulin (Kopelman *et al.*, 1980). Thus, in obese males, the decrease in SHBG results in less estrogen bound, ending up in more biologically active, free estrogen. In addition to the conversion of

testosterone to estrogen in obese patients, the decreased ability of SHBG to sustain homeostatic levels of free testosterone also contributes to abnormal testosterone levels (Du Plessis *et al.*, 2010, Jensen *et al.*, 2004). This failure to maintain the normal levels of testosterone may magnify the negative feedback of elevated total estrogen levels. Thus, obese men present higher levels of estrogens and decreased levels of total testosterone. Furthermore, it has already been shown that obesity and infertility are genetically related. In fact, twenty-one genes linked with this metabolic disorder were also associated with human infertility and reproduction (Butler *et al.*, 2015). However, if we have a closer look to the identified genes, it is not surprising that most of them is related with endocrine regulation, as is the case of leptin, follicle-stimulating hormone and insulin-like growth factor 1 receptors (Butler *et al.*, 2015).

Obesity is also intimately linked with the consumption of high energy diets. Due to FFA overload, testicular glycolysis is disrupted, leading to a deficient nurture of developing germ cells. Besides, lipid metabolism is also compromised in Sertoli cells, inducing a deficient germ cell membrane remodeling and structure. All of these effects in testicular environment, due to high energy diet consumption, lead to a decrease in semen quality (Rato *et al.*, 2014). Moreover, a major factor that contributes to obesity-related sperm quality problems is the increased oxidative stress and lipid peroxidation (Jones *et al.*, 1979). Indeed, it was observed that spermatozoa from infertile obese men presented increased oxidative injury and DNA fragmentation, which reflect the abnormal oxidative state of the testicular microenvironment and efferent duct system (Aitken *et al.*, 1998, Kort *et al.*, 2006). Moreover, it was found that sperm cells from obese individuals have decreased mitochondrial membrane potential. This alteration anticipates phosphatidylserine externalization to the external leaflet of the plasma membrane, a potentially reversible signal, which starts the cascade of events leading to final DNA fragmentation and possibly apoptosis (La Vignera *et al.*, 2012). Thus, although the information about the effects of obesity on sperm parameters varies, it is clear that at least increases the possibility of sperm cells having oxidative injury. Thus, and as previously stated, the antioxidant therapy may be beneficial in cases of obesity-induced oxidative stress in the male reproductive tract (Ross *et al.*, 2010).

In the last years, and due to the increase of both DM and infertility, the attention given to the mechanisms that may interconnect these disorders has increased. It is now widely accepted that individuals with prediabetes and diabetes have a significant decrease in sperm quality (Boeri *et al.*, 2019, La Vignera *et al.*, 2009). This may be due to several factors, namely increased sperm nuclear DNA fragmentation or mitochondrial DNA (mtDNA) damage (Agbaje *et al.*, 2007); impairment of sperm viability, motility and morphology (Oliveira *et al.*, 2015, Rato *et al.*, 2013); or even to decreased fecundity capacity (Scarano *et al.*, 2006). In fact, it was shown that prediabetes decreases the antioxidant potential of testicular tissue, while increases lipid peroxidation and protein carbonylation (Oliveira *et al.*, 2015, Rato *et al.*, 2014). Likewise, individuals with prediabetes had a decrease in testicular peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and sirtuin 3 levels (Rato *et al.*, 2014). The decrease of PGC-1α and sirtuin 3 is known to favor oxidative stress, due to an unbalance between antioxidant defenses and ROS (Jing *et al.*, 2011). Thus, this may lead

to the observed DNA damage. Moreover, DM also affects hormonal levels. Indeed, a significant decrease in both luteinizing hormone and follicle-stimulating hormone levels was described in the plasma of individuals with diabetes (Scarano *et al.*, 2006). Remarkably, insulin injections can restore luteinizing hormone to normal levels (Benitez and Perez Diaz, 1985, Hutson *et al.*, 1983).

T2DM also induces an increase in both serum and intratesticular testosterone levels (Rato *et al.*, 2015). Since testosterone is produced by Leydig cells, this may be related with the finding that Leydig cells presented abnormal morphology in diabetic individuals, with lipid droplets in their cytoplasm (Cameron *et al.*, 1985). In the same study, it was also reported that Sertoli cells presented an extensive vacuolization and a high degree of degeneration (Cameron *et al.*, 1985). Although sperm morphology was normal, these modifications have drastic consequences on testicular cells metabolism and consequently, on the adequate nurture of germ cells. In fact, DM led to a reduction in the production of lactate by testicular cells through a decrease in lactate dehydrogenase activity (Rato *et al.*, 2015). Furthermore, DM was also found to be related with ionic modifications in both testis and epididymis (Bernardino *et al.*, 2013). In the testis, there was an increase in the transcript levels of several bicarbonate transporters in prediabetic rats. On the other hand, prediabetes also altered the protein expression of some bicarbonate transporters in the epididymis, suggesting an alteration in bicarbonate homeodynamics which may lead to an inadequate environment for sperm storage (Bernardino *et al.*, 2013). Thus, DM impacts sperm viability and, consequently, male reproductive potential. There are several studies indicating that diabetic patients have impaired sperm parameters, namely decreased motility (Mulholland *et al.*, 2011). Concerning assisted reproduction techniques, fertilization rates and embryo quality did not differ from non-diabetic couples, but pregnancy rates were lower in couples with a diabetic male (Mulholland *et al.*, 2011). Of note, diabetic male individuals also present sexual disorders, such as retrograde ejaculation, decreased libido and erectile dysfunction (Meneses *et al.*, 2015b). Despite all that is already known, further studies are needed to deeply understand the molecular mechanisms that interconnect DM and infertility.

Testicular cells metabolism: the relevance of glucose homeostasis

Glucose is crucial for spermatogenesis, since it is the precursor for the lactate produced by Sertoli cells that is then used by developing germ cells to suppress their energetic needs (Mita and Hall, 1982, Riera *et al.*, 2009). It has been described that glucose deprivation led mammalian cells to increase glucose uptake, showing that glucose may regulate its own transport and metabolism (Dias *et al.*, 2013, Klip *et al.*, 1994). This regulation includes altered expression of glucose transporters, which is increased in response to glucose deprivation. In fact, the expression of GLUT1 and GLUT3 was found to be increased through the activation of PI3K/Akt, AMPK and p38MAPK dependent pathways (Riera *et al.*, 2009). Besides, insulin and some cytokines and growth factors differentially modulate the expression of those glucose transporters (Berg *et al.*, 2002, Oliveira *et al.*, 2012). Consequently, Sertoli cells can increase the uptake of glucose in order to produce the adequate amounts of lactate. This will fulfill the needs of developing germ cells even in glucose deprivation conditions (Riera *et al.*, 2009). These fluctuations in glucose and insulin concentration lead to alterations in Sertoli cells

metabolism that can impair the development of germ cells and consequently male fertility. However, besides the relation between insulin and glucose, insulin-like growth factor 1 (IGF-1) influences glucose transport in testicular cells. In fact, an overexpression of IGF-1 results in increased developing germ cells apoptosis and thus, abnormal spermatogenesis and reduced male fertility potential (Froment *et al.*, 2004). In sum, glucose homeostasis is essential for male fertility, or else may end-up in subfertility/infertility. There are several homeostasis pathways that cause alterations in testicular cells metabolism. Yet, these cells present some important compensatory mechanisms that allow the preservation of energy necessities of germ cells.

Insulin-Degrading Enzyme

Insulin-degrading enzyme (IDE), also known as insulysin, is greatly responsible for insulin cleavage and inactivation (Pivovarova *et al.*, 2016). It may be found in several cellular compartments, namely in the cytosol, peroxisomes, endosomes, mitochondria and cell membrane (Leissring *et al.*, 2004, Vekrellis *et al.*, 2000). IDE is highly conserved between species. In humans, IDE is ubiquitously expressed independently of the sensitivity of the cells to insulin (Pivovarova *et al.*, 2016). Furthermore, its levels and conformation may be controlled by several signals, including cellular stress, glucagon, and FFA (Tundo *et al.*, 2013, Wei *et al.*, 2014). This indicates that IDE has a multifunctional role and that it is finely tuned depending on the state of the cell. In fact, IDE was found to have a heat shock protein-like behavior and to interact with the ubiquitin-proteasome system (Tundo *et al.*, 2013). IDE not only cleaves insulin, but also other peptides of assorted sequence, namely amylin and glucagon, showing to be a player in the regulation of carbohydrate metabolism (Duckworth and Kitabchi, 1974, Maianti *et al.*, 2014). Moreover, IDE also degrades amyloid ß-protein (Farris *et al.*, 2003, Leissring and Selkoe, 2006) thus being closely related to Alzheimer's disease. Actually, IDE was already hypothesized to be one of the links between Alzheimer's disease and T2DM (Pivovarova *et al.*, 2016). By degrading insulin, IDE has a crucial role in insulin metabolism and in inhibiting its translocation and accumulation in the cell nucleus (Authier *et al.*, 1996, Harada *et al.*, 1993). Thus, loss-of-function mutations of IDE cause hyperinsulinemia (Farris *et al.*, 2004), one of the main features of prediabetes and T2DM. Inhibitors of IDE were already hypothesized as possible treatment for those disorders (Costes and Butler, 2014) but as IDE degrades other substrates besides insulin, this is a very complex topic. In fact, and due to its impact in insulin and amyloid ß-protein levels, there are several studies evaluating the importance of IDE towards the liver (Harada *et al.*, 1993, Wei *et al.*, 2014), the pancreas (Fernández-Díaz *et al.*, 2019) and the nervous system (Vekrellis *et al.*, 2000). There are no reports about the direct impact of IDE on male reproductive system. However, as IDE is crucial for insulin homeostasis, and the latter is, in turn, essential for a proper reproductive function (Oliveira *et al.*, 2012), IDE may have an important role in determining male reproductive function.

The decrease in fertility rates are in part suggested to be associated with the increase of metabolic disorders. As discussed, men with metabolic diseases present numerous dysfunctions in their reproductive system that can lead to infertility or, at least, subfertility. Therefore, it is of great

importance to unveil the mechanisms induced by metabolic disorders that result in the impairment of the male reproductive tract. Within the seminiferous tubules, insulin regulation and glucose metabolism are crucial for male reproductive function since several essential metabolites for developing germ cells, as lactate or acetate, are produced from glucose. As discussed, individuals with metabolic disorders present impaired testicular metabolism that may end-up in reduced sperm quality and consequently in male subfertility/infertility. The information concerning metabolic diseases and their underlying mechanisms in whole body glucose/insulin homeostasis is rapidly shifting. Regrettably, the knowledge in what concerns to testicular metabolism remains scarce. It is pivotal to unveil the way whole body metabolic state may influence testicular metabolic status.

II. AIMS AND HYPOTHESES

Given the significant increase in the prevalence of obesity and prediabetes/T2DM worldwide, it is crucial to understand the pathogenesis of these metabolic diseases and its effects on different organs and systems. The alterations that occur in metabolic diseases, namely in glucose and insulin homeostasis, are known to have a great impact on several organs. In this work, we focus in organs affected by prediabetes and obesity that are usually neglected, namely due to the absence of symptoms – the liver and the male reproductive system.

The liver is a metabolic organ responsible for whole-body energy metabolism. Any disruption of hepatic metabolism may contribute to the onset and progression of metabolic-related diseases. Specifically, increased hepatic ectopic lipid accumulation may contribute to the progression or the development to steatohepatitis and fibrosis, leading to the metabolic complications of NAFLD.

The formation of spermatozoa is an overly complex, energy-demanding, and tightly regulated process. It is known that glucose and insulin levels have an impact on the reproductive potential. However, the effects of primary hyperinsulinemia are not fully understood.

The overall aim of this dissertation was to investigate the homeostatic regulation of insulin, glucose and lipid metabolism in altered metabolic states such as obesity and IR. The studies were designed to explore the molecular mechanisms underlying the development of obesity and IR in the context of glucose metabolism.

In detail, the following hypotheses and aims were designed:

Hypothesis 1: Diet composition differentially affects the metabolome of insulin-sensitive organs.

Aim: To determine the metabolome of the liver, muscle and brown adipose tissue and the metabolic crosstalk between these organs. In addition, we aimed to determine the hepatic protein levels of PON1 after high-fat or high-fructose diet.

Hypothesis 2: PON1 deletion exacerbates diet-induced obesity, hepatosteatosis and IR.

Aim: To determine the physiological role of PON1 in overall glucose homeostasis and hepatic glucose and lipid metabolism.

Hypothesis 3: PON1 paraoxonase activity (POase) and Apolipoprotein J (ApoJ) levels can be used as co-biomarkers in early stages of dysmetabolism.

Aim: Determine if POase activity and ApoJ levels improve the stratification of a population in relation to dysmetabolism, helping to distinguish phenotypes with distinct pathophysiological meaning.

Hypothesis 4: IDE may be a key player in insulin dysfunction-associated male infertility.

Aim: To determine the impact of primary hyperinsulinemia in male reproductive potential due to *Ide* deletion.

III. GENERAL METHODOLOGY

The methodology used throughout our studies will be described in detail in Chapter IV – Results. However, some general methodological considerations should be addressed beforehand.

Mouse Studies

During this work we used different animal models to study prediabetes and associated co-morbidities such as insulin dysregulation, obesity, and NAFLD. In the last years, an extensive number of animal models have been generated to study these disorders. Generally, the approach to obtain these models goes from dietary interventions to genetic modifications, surgical or chemical interventions or even a combination of more than one of the previous.

The majority of research studies on NAFLD are performed using models of spontaneous genetic origin, or experimentally induced by diet (e.g. high-fat diet, methionine and choline-deficient diet or high-fructose diet).

High-fat and High-fructose diet induced insulin resistance in C57Bl6/J mice

For the study of a lipid or fructose-enriched diet on PON1 levels we used C57Bl6/J mice, a widely used strain for the study of insulin resistance, obesity and NAFLD. In this case, we used two different diets in addition to the normal chow diet (composition in Annexes, Figure 32): a high fat diet (composition in Annexes, Figure 33):, selected since it leads to a metabolic phenotype similar to what is observed in humans (obesity, insulin resistance, hyperinsulinemia, glucose intolerance and hyperlipidemia) (Jahn *et al.*, 2019); and a high-fructose diet, since it is a major component of the most commonly used sweeteners and it was already demonstrated to exacerbate hepatic fat deposition, inflammation and IR (Jegatheesan and De Bandt, 2017).

Genetically Modified Mouse Models

For the study of hyperinsulinemia impact on male reproductive function, we have used a mouse model lacking IDE. As the absence of IDE *per se* is known to lead to hyperinsulinemia and prediabetes (Farris *et al.*, 2004), we used normal chow diet. On the contrary, to study the role of PON1 in regulating glucose and lipid metabolism we used a genetically modified mouse model for the *Pon1* gene under normal chow, high-fat or high-fructose diets. To create this model, the exon 1 was disrupted with a targeting vector containing a neomycin-resistance gene expression cassette (Shih *et al.*, 1998). Consequently, the resultant mice do not have paraoxonase activity in plasma and PON1 is not detected by western blot.

Human Studies

PREVADIAB2

The PREVADIAB study was the first study of diabetes prevalence in Portugal (Gardete-Correia *et al.*, 2010). It started in 2008 with PREVADIAB1, in which 5167 individuals were included. These individuals had between 20 and 79 years old and were representative of the Portuguese population distribution.

PREVADIAB2 is a follow-up study of PREVADIAB1, that aimed at evaluating the progression to prediabetes or diabetes of non-diabetic individuals of PREVADIAB1. In PREVADIAB2, 1088 individuals were included. After an overnight fasting, an oral glucose tolerance test was performed, along with blood analysis. Additionally, anthropometric data was collected. Insulin secretion and clearance was also evaluated through the analysis of insulin and C-peptide levels. With the samples available in the biobank, we intend to verify if ApoJ levels and PON1 activity have any correlation with the glycemic state of the individuals.

As mentioned in the beginning of this section, the detailed experimental procedures for each individual study that composes this dissertation, will be presented in the corresponding results section.

IV. RESULTS

This chapter was adapted from the published work:

Meneses MJ, Borges DO, Dias TR, Martins FO, Oliveira PF, Macedo MP, Alves MG (2019). Knockout of insulin-degrading enzyme leads to mice testicular morphological changes and impaired sperm quality. Molecular and Cellular Endocrinology. 486:11-17.

Metabolic crosstalk of hepatic, muscular and brown adipose tissues after high fat or high fructose diets

Abstract

In the last years, the consumption of ready-made meals, rich in carbohydrates, saturated fat and cholesterol has radically increased. Changes in dietary habits have contributed to the increasing prevalence of obesity and associated metabolic disorders, such as non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes *mellitus* (T2DM). These metabolic disorders are known to affect several organs, due to the overload of free fatty acids and glucose and inter-organ crosstalk. Paraoxonase-1 (PON1), an enzyme mainly produced in the liver, has been linked to the prevention of atherosclerosis, attributable to its antioxidant and anti-inflammatory potential. Herein, we hypothesized that high-fat and high-fructose diets differentially affect the metabolome and the crosstalk of insulin-sensitive organs and that PON1, as an antioxidant protein, has a role in protecting against prediabetes onset. For this, we have studied the impact of 12 weeks of high-fat/sucrose (HFat) or high-fructose (HFruct) feeding on C57Bl/6J mice. The metabolome of liver, muscle and brown adipose tissue (BAT) was analyzed. HFat diet fed mice developed glucose intolerance and insulin resistance while HFruct diet fed mice had glucose tolerance similar to Chow animals. However, both HFat and HFruct fed mice affected the metabolome of liver, muscle and BAT. However, HFat fed mice had a more distinct metabolome compared to the metabolome of Chow fed mice that HFruct diet fed animals metabolome. Moreover, hepatic PON1 levels are decreased when dietary contents in fructose and lipids are increased. These results illustrate that HFat and HFruct differentially affect the metabolome of liver, muscle and BAT and, consequently, differentially affect the crosstalk between these tissues.

Keywords: Prediabetes; Non-Alcoholic Fatty Liver Disease; High-Fat Diet; Fructose; Metabolomics; Muscle; Paraoxonase-1; Liver; Muscle; Brown Adipose Tissue

Introduction

Obesity is caused by excessive and abnormal accumulation of body fat bringing several adverse health effects (Fan *et al.*, 2017). Although the awareness for this health and social problem has increased in the last years, its prevalence continues to increase dramatically. In fact, by 2025, it is expected that global obesity prevalence will reach 18% in men and exceed 21% in women. Moreover, severe obesity will surpass 6% in men and 9% in women (NCD Risk Factor Collaboration, 2016). Although genetic background influences obesity development (Grarup *et al.*, 2014), the vast majority of obesity cases are due to factors that arose in western countries, namely the excessive consumption of highly-processed and energy dense foods, frequently combined with a sedentary lifestyle.

Adipose tissue hypertrophy, resultant from these lifestyle habits, leads to metabolic and homeostatic dysfunctions (Tchernof and Després, 2013). In fact, obesity increases the risk of comorbidities such as hypertension, T2DM (Colditz *et al.*, 1995), cardiovascular disease (Willett *et al.*, 1995), dyslipidemia (Pascot *et al.*, 2001) and NAFLD. The latter is characterized by the presence of macrovesicular steatosis in ≥5% of hepatocytes, in individuals with no causes for secondary hepatic accumulation, as is the case of abusive alcohol consumption and hereditary disorders (Chalasani *et al.*, 2012). NAFLD is the most common cause of chronic liver disease in Western countries, having a reported prevalence of 6-35% worldwide (Bellentani, 2017). Although the molecular mechanisms that lead to NAFLD continue to be discussed, it is known that it is a consequence of several factors, namely: i) an increased incursion of free fatty acids from insulin-resistant adipose tissue; ii) impaired processing of dietary lipids in the liver or lipid export from the hepatocytes; iii) increased *de novo* lipogenesis in the liver (Bosserhoff and Hellerbrand, 2011). The overload of lipids affects not only the liver, but other metabolic organs such as the muscle or brown adipose tissue. Considering inter-organ crosstalk dysfunctions in one of these organs will impact on others, namely in cases of insulin resistance (De Bandt *et al.*, 2018, Scheja and Heeren, 2016).

PON1 was firstly found due to its ability to hydrolyze the insecticide paraoxon, although its physiological substrate remains to be unveiled. It is known to have a role in atherosclerosis, by preventing LDL oxidation and promoting cholesterol efflux from macrophages, thus preventing foam cell formation (Aviram and Rosenblat, 2004). In fact, PON1 is mainly expressed in the liver and released into the circulation, where it is found in HDL particles. The role of hepatic PON1 in the molecular mechanisms of diet-induced obesity remains to be determined. We hypothesized that lipid and fructose content of the diet differentially affect the metabolome of insulin-sensitive organs and that PON1, as an antioxidant protein, has a role in protecting against prediabetes onset. Our aim is to evaluate the metabolome of insulin sensitive organs, as is the case of the liver, muscle and brown adipose tissue and the metabolic crosstalk between these organs. Furthermore, we aim to determine the hepatic protein levels of PON1 after high-fat or high-fructose feeding.

Materials and Methods

Chemicals

Bicinchoninic acid (BCA) Protein Assay Kit was purchased from Thermo Scientific (Whalthan, MA, USA). Dried milk was purchased from Nestlé (Vevey, Switzerland). Amersham ECL was purchased from GE Healthcare (Weßling, Germany). NZYColour Protein Marker II, agarose, NZYDNA Ladder V, and Supreme NZYTaq II 2x Green Master Mix were purchased from NZYTech (Lisbon, Portugal). Fructose was purchased from Enzymatic (Loures, Portugal). Immobilon-P PVDF Membrane was purchased from Merck Millipore (Massachusetts, USA). Kits for Triglycerides and Cholesterol determination were purchased from Spinreact (Girona, Spain). Quantification of free fatty acids was done using a kit from Wako Diagnostics (CA, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless stated otherwise.

Animals

Experiments were performed using male C57Bl6/J mice that were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. Animals were randomly divided in three groups (n=7/group) and fed different diets from 6 to 18 weeks of age: normal chow diet with 11.50% calories from fat, 26.93% from protein and 61.57% from carbohydrates (Chow group; RM3A(P), Special Diets Services, Witham, Essex, UK); high-fat diet with 58% of calories from fat, 16.4% from protein and 25.5% from carbohydrates (HFat; D12331, Research Diets, New Brunswick, NJ) or high fructose diet (HFruct; FRUC-00T-500, Enzymatic, PT; 35% w/v in drinking water). Mice were monitored weekly for body weight, blood glucose levels, as well as for distress signals. The experimental procedures received prior approval by the Ethics Committee of the NOVA Medical School and by the Directorate-General for Food and Veterinary that regulates the animal care and use in research. All procedures followed Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the European laws (Directive 2010/63/EU) that rule the use of animals in research.

Metabolic Measurements

Mice were weighed at 6 weeks and weekly thereafter. Blood glucose levels were measured using a Contour Next glucose meter (Bayer, Leverkusen, Germany). Food and caloric intake were also measured. For glucose tolerance tests, mice were fasted overnight at 17 weeks of age, and blood glucose levels were measured before and 15, 30, 60, 90 and 120 min after intraperitoneal injection of glucose (2.0 g/kg of body weight). For insulin tolerance tests, at 17 weeks of age mice were fasted for 5 h, and blood glucose levels were measured before and 15, 30, 60, 90 and 120 min after intraperitoneal injection of human insulin (0.75 UI/kg of body weight; Actrapid, Novo Nordisk). Area under the curve (AUC) was calculated using the trapezoidal rule for glucose data (Andrikopoulos *et al.*, 2008), respectively.

Insulin signaling studies

Mice were fasted overnight and intraperitoneally injected with human insulin (10 UI/kg of body weight; Actrapid, Novo Nordisk) or saline and sacrificed 10 min later. Organs were harvested, snap frozen and stored at -80°C until analysis. Liver was homogenized in lysis buffer (in mM: 20 Tris pH 7.5, 5 EDTA, 10 $Na_4P_2O_7$, 100 NaF, 2 Na_3VO_4) with 1% NP-40 and protease inhibitors (Roche, Switzerland). Tissue lysates (20 µg protein) were mixed with Laemmli sample buffer (in %: 1.5 Tris, 20 glycerol, 4.1 SDS, 2 ß-mercaptoethanol, 0.02 bromophenol blue, pH 6.8) and denatured for 10 min at 95°C. Proteins were fractionated in 10% polyacrylamide gels. The proteins were transferred from gels to previously activated polyvinylidene difluoride (PVDF) membranes in TransBlot Turbo (Bio-Rad Laboratories, Hemel Hempstead, UK) and then blocked for 1 h in a 5% non-fat milk solution at room temperature. The membranes were incubated overnight at 4°C with the primary antibodies listed in Table 2 and incubated with secondary antibodies for 1 h. Either glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or ß-actin were used as loading controls. Membranes were reacted with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare), read with the Chemidoc and quantified using ImageLab (Bio-Rad Laboratories).

Table 2 – List of antibodies used to evaluate the impact of high-fat and high-fructose diets on the insulin signaling pathway.

Antibody	Host Specie	Molecular Weight	Dilution	Vendor	Catalog #
InsR-α	Rabbit	95 kDa	1:1000	Cell Signaling	74118
pInsR	Rabbit	95 kDa	1:1000	ThermoFisher Scientific	44-806G
Akt	Rabbit	60 kDa	1:1000	Santa Cruz Biotechnology	sc-7582
pAkt $_{S473}$	Rabbit	60 kDa	1:1000	Cell Signaling Technology	4060
pAkt $_{T308}$	Rabbit	60 kDa	1:1000	Cell Signaling Technology	4056
PON1	Rabbit	43 kDa	1:2000	Abcam	ab126597
GAPDH	Mouse	37 kDa	1:10000	Ambion	AM4300
ß-Actin	Mouse	42 kDa	1:5000	Cell Signaling Technology	3700
Mouse	Goat	–	1:5000	Santa Cruz Biotechnology	sc-2031
Rabbit	Goat	–	1:5000	Santa Cruz Biotechnology	sc-2030

InsR-α – Insulin receptor alpha; pInsR – phosphorylated insulin receptor; Akt – protein kinase B; pAkt – phosphorylated protein kinase B; PON1 – paraoxonase-1; GAPDH - glyceraldehyde-3-phosphate dehydrogenase.

Liver histology and lipid assay

Liver was fixed with 10% formalin solution, embedded in paraffin and cut in 4 µm sections. These sections were then stained with hematoxylin and eosin for the characterization of liver morphology and lipid content. Hepatic lipids were extracted as previously described (Matyash *et al.*, 2008). Briefly, approximately 250 mg of frozen tissue was rapidly mixed with high performance liquid chromatography (HPLC)-grade methanol (4.6 mL/g) followed by methyl-tert-butyl ether (MTBE) (15.4 mL/g). The mixture was placed in a shaker for 4 h and then centrifuged at 13,000 .*g* for 10 min.

The liquid fraction was collected, and phase separation was induced by adding 1 mL of distilled water and letting it rest at room temperature for 10 min. The liquid was then centrifuged for 10 min at 1000 *.g*. The organic phase, which contained the lipids, was separated and dried under nitrogen gas in a glass vial protected from light. It was then dissolved in butanol/(Triton X-100:methanol [2:1]). Hepatic total cholesterol and triglyceride contents were determined by enzymatic method (SpinReact).

Quantitative real-time polymerase chain reaction (PCR)

The extraction of liver total ribonucleic acid (RNA) was performed using TRIzol (Invitrogen) and the concentrations were determined by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). Total RNA was reversely transcribed to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, California, USA). Quantitative real-time polymerase chain reaction (qPCR) was performed to evaluate the abundance of messenger RNA (mRNA) coding for GK, G6Pase, PEPCK, ChREBP, SREBP2, ELOVL2, CD36, SCD1, ß-actin and ß-2-microglobulin using SYBR™ Green PCR Master Mix (Applied Biosystems) in an ABI 7500 (Applied Biosystems). Specific exon-exon spanning primers were designed for the amplification of the target and housekeeping transcripts (Table 3). ß-actin and ß-2-microglobulin transcript levels were used to normalize gene expression levels. Fold variation in gene expression levels was calculated with the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Table 3 – Primer sequences and cycling conditions for polymerase chain reactions to evaluate the impact of high-fat and high-fructose diets in gene expression. Primer sequences of glucokinase (GK), glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), carbohydrate-responsive element-binding protein (ChREBP), sterol regulatory element-binding protein 2 (SREBP2), elongation of very long chain fatty acids protein 2 (ELOVL2), cluster of differentiation 36 (CD36), stearoyl-CoA desaturase (SCD1), ß-actin and ß-2-microglobulin.

Gene	Sequence 5' -3'	Annealing Temperature	Cycles
Glucokinase (NM_010292.5)	Fwd: GCTGGTACGACTTGTGCTG Rvs: TGGACACGCTTTCACAGG	60°C	35
G6Pase (NM_008061.4)	Fwd: ATGAACATTCTCCATGACTTTGGG Rvs: GACAGGGAACTGCTTTATTATAGG	58°C	35
PEPCK (NM_011044.3)	Fwd: TGAAAGGCCGCACCATGTAT Rvs: GCACAGATATGCCCATCCGA	60°C	35
ChREBP (NM_001359237.1)	Fwd: CTGGGGACCTAAACAGGAGC Rvs: GAAGCCACCCTATAGCTCCC	60°C	35
SREBP2 (NM_033218.1)	Fwd: GCGTTCTGGAGACCATGGA Rvs: CACAAGTTGCTCTGAAAACAAATCA	60°C	35
Elovl2 (NM_001311121.1)	Fwd: CCTGCTCTCGATATGGCTGG Rvs: AAGAAGTGTGATTGCGAGGTTAT	60°C	35
CD36 (NM_001159558.1)	Fwd: GATGACGTGGCAAAGAACAG Rvs: TCCTCGGGGTCCTGAGTTA	58°C	35
SCD1 (NM_009127.4)	Fwd: CGCCCCTACGACAAGAACAT Rvs: CTCAGAAGCCCAAAGCTCAG	60°C	35
ß-actin (NM_007393.5)	Fwd: CGTGAAAAGATGACCCAGATCA Rvs: CACAGCCTGGATGGCTACGT	60°C	35
ß-2-microglobulin (NM_009735.3)	Fwd: TCTCACTGACCGGCCTGTAT Rvs: CAGTCTCAGTGGGGGTGAAT	60°C	35

Metabolites extraction

To extract liver, muscle and brow adipose tissue metabolites, the different tissues were homogenized in glass vials in a mixture of methanol and chloroform (2:1). After sonication in ice for 15 min, chloroform and water (1:1) were added and samples were centrifuged at 10,000 .*g* for 15 min at 4°C. The supernatant was then evaporated using a flow of nitrogen and dissolved in D_2O phosphate buffer (0.2 M, pH = 7) for proton nuclear magnetic resonance (^{1}H-NMR) analysis.

Nuclear Magnetic Resonance spectroscopy

^{1}H-NMR spectra of the polar extracts were acquired using a Varian Inova 600 MHz (14.1 T) spectrometer equipped with a 3 mm QXI probe with a z-gradient. ^{1}H-1D noesy experiments with water presaturation were acquired at 298 K (7.2 kHz spectral width, 0.1 s mixing time, 4 dummy scans, 4 s relaxation delay with 3 s of water presaturation, 90º pulse angle, 3 s acquisition time and a minimum of 128 scans; typical spectra obtained for the tissues are represented as supplementary data - Figure 34). To further spectral assignment two-dimensional spectra (TOCSY) were acquired using sweep width of 5.4 kHz in both dimensions, 48 transients and 400 and 1024 points in t1 and t2 dimensions, respectively. Spectra were treated by multiplying FIDs with exponential window function (line broadening of 0.3 Hz) and were zero filled to 64 k points prior to Fourier transformation using TopSpin (Bruker Biospin, Karlsruhe, Germany). It was processed by applying qsine window function and zero filled to 2048 point in both dimensions. The comparison of 1D and 2D spectra with reference spectra and public databases such as HMDB allowed to assign the peaks (Wishart *et al.*, 2007). Metabolites were identified according to metabolomics standards initiative guidelines for metabolite identification (Sumner *et al.*, 2007).

Multivariate analysis of NMR data

Processed 1D noesy spectra were bucketed using one-point bucket (0.6-9.0 ppm, with signal-free, water and fumarate regions excluded). Data matrix was built in Amix Viewer (BrukerBiospin, Rheinstetten). Icoshift algorithm (Savorani *et al.*, 2010) was used to align bucketed spectra and total area integral normalization was applied to account for the variations in the overall sample concentrations. Multivariate statistical analysis was applied on unit variance scaled matrix (SIMCA 14, Umetrics, Sweden). Principal component analysis (PCA) was used to provide information on global data structure, and partial least squares discriminant analysis (PLS-DA) was used to assess group separation and to identify the main metabolites that contribute to the group discrimination. PLS-DA models were validated by 7-fold cross-validation and permutation test (n=100) to provide the qualitative measure of predictive power (Q2) and to assess the degree of fit to the data (R2). The corresponding PLS-DA loadings were obtained by multiplying the loading weight factors (w) by the standard deviation of the respective variable and were color-coded according to variable importance in the projection (VIP). All differentially expressed metabolites for each of the diets in each tissue were used to identify the most relevant metabolic pathways affected by diet using MetaboAnalyst (Xia and Wishart, 2016).

Statistical Analysis

Experimental data are shown as mean ± standard error of mean (SEM). Unless stated otherwise, statistical analysis was performed using one-way ANOVA in Graphpad Prism 8 (GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered significant.

Results

HFat diet fed mice develop glucose intolerance, insulin resistance and non-alcoholic fatty liver disease

After a 12 week period of diet intervention, mice subjected to an HFat diet had increased body weight gain (37.90 ± 1.06 g) compared to Chow diet fed mice (28.14 ± 0.57 g; Figure 7A). Conversely, HFruct diet fed mice had decreased body weight (24.86 ± 0.76 g). HFat diet fed mice were glucose intolerant, as observed by failure to decrease blood glucose levels after administration of a glucose bolus (Figure 7B). HFruct diet fed mice displayed normal glucose tolerance but decreased insulin sensitivity, due to the inability to decrease blood glucose levels after an insulin bolus (Figure 7B-C). In fact, after 12 weeks of diet, HFat diet fed mice had marked hyperglycemia (136 ± 12 mg/dL), compared to Chow diet fed mice (98 ± 8 mg/dL, Figure 7D), while HFruct diet fed mice were normoglycemic (102 ± 10 mg/dL). HFruct diet fed mice had decreased water intake, concomitant with decreased food intake, compared to Chow diet fed mice. However, this was not reflected in a decrease in caloric intake, due to supplementation of drinking water with fructose (Figure 7E-F). Caloric intake was increased in HFat diet fed mice, compared to Chow diet fed mice (Figure 7G).

Obesity is associated with ectopic fat deposition in parenchymatous organs such as the liver. To evaluate this, hepatic triglycerides and cholesterol were measured. Liver content in both triglycerides and cholesterol was increased in HFat diet fed mice (35.08 ± 2.35 mg/g and 3.45 ± 0.19 mg/g, respectively) but unchanged in HFruct diet fed mice (26.52 ± 0.94 mg/g and 2.66 ± 0.03 mg/g, respectively; Figure 7H-I) when compared to Chow diet fed mice (25.35 ± 1.57 mg/g and 2.85 ± 0.14 mg/g, respectively). The analysis of histological sections demonstrated that HFat diet fed mice developed NAFL, whereas HFruct diet fed mice had mild hepatic degeneration (Figure 7J). Insulin-stimulated glucose utilization relies on an effective insulin signaling pathway. Moreover, insulin is known to impact the transcription of several lipid and carbohydrate metabolism-related genes (Patti, 2004). Therefore, the protein levels of the insulin pathway-related components insulin receptor (InsR) and Akt in the liver of the mice were determined. HFat diet fed mice presented a decrease in InsRα levels and HFruct diet fed mice presented a further decrease, after 12 weeks of diet exposure. However, the activation of the pathway, observed by the ratio between phospho-insulin receptor (pInsR), did not present any differences between the groups (Figure 7K-L). When analyzing Akt phosphorylation levels, there was a marked reduction in both T308 and S473 in both HFat and HFruct diet fed groups, demonstrating that both diets negatively impact on the activation of the insulin signaling pathway (Figure 7K-L).

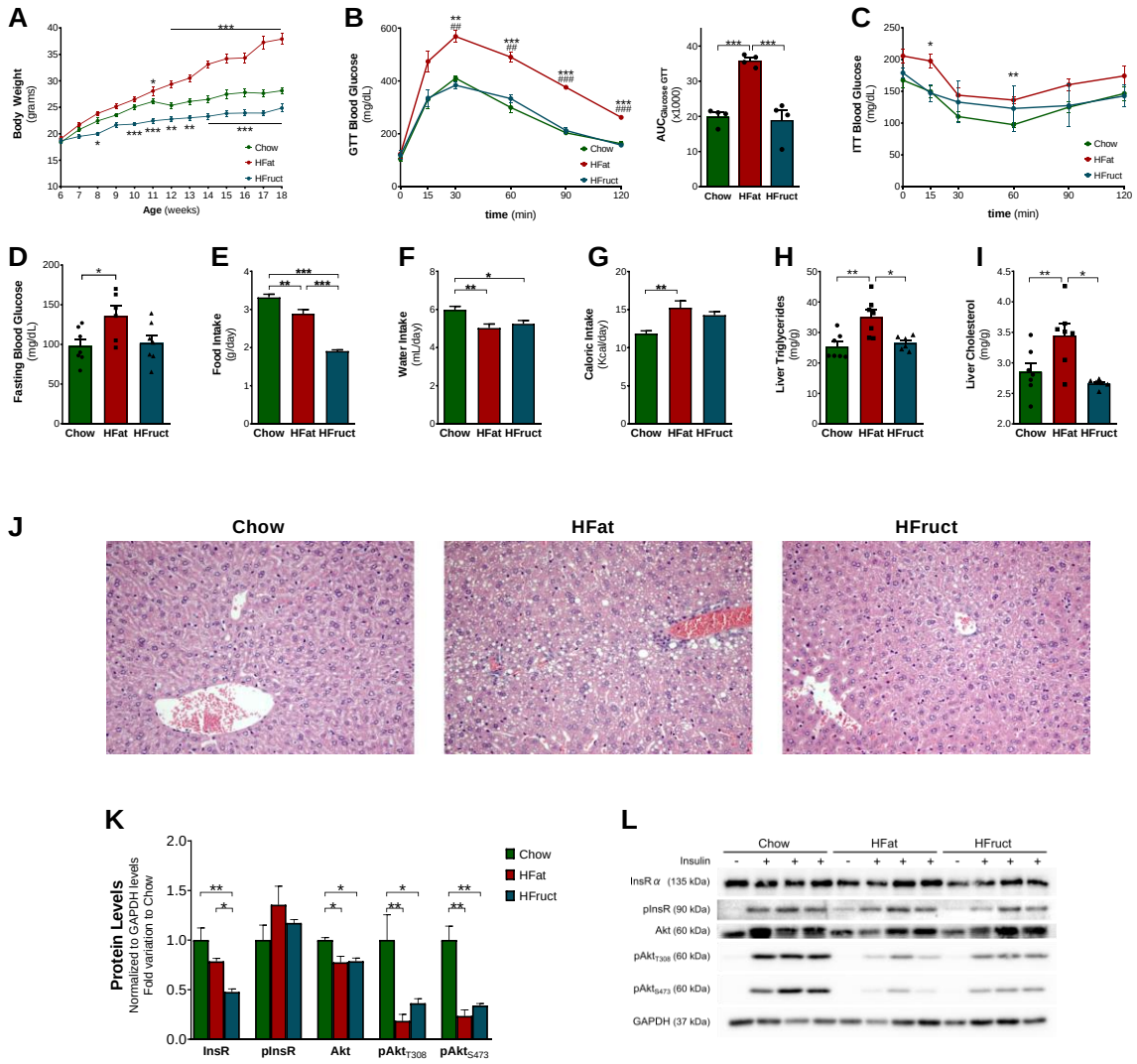

Figure 7 - Effects of HFat and HFruct diets on metabolic parameters, glucose homeostasis, hepatic lipid deposition and insulin signaling pathway in C57Bl/6J male mice. A – Body weight, B – Intraperitoneal glucose tolerance test (ipGTT) and the respective area under the curve (AUC), C – Insulin tolerance test (ITT); D – Fasting blood glucose; E – Food intake; F - Water intake; G – Caloric intake; H – Hepatic triglycerides; I - Hepatic cholesterol levels; J – Representative images of hematoxylin and eosin stained liver sections; K - Chow, high-fat (HFat) and high-fructose (HFruct) hepatic protein levels of Insulin Receptor α (InsR), phospho-Insulin Receptor (pInsR; normalized to InsR levels); protein kinase B (PKB/Akt) and its phosphorylation sites ($pAkt_{T308}$ and $pAkt_{S473}$; normalized to Akt levels) normalized to GAPDH levels and represented as fold variation to Chow group; B – Representative images of western blot for the described antibodies. Data are presented as mean ± SEM for 3-7 mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; In panels B: ## $p<0.01$ vs. HFruct, ### $p<0.001$ vs. HFruct.

Hepatic carbohydrate, lipid and amino acid metabolism is impaired by HFat and HFruct diets

The liver is a key organ for the maintenance of normal glucose homeostasis. However, this homeostasis is impaired in insulin resistant states, thus we evaluated the intracellular levels of key metabolites in the fasting state, as well as the mRNA expression of some glucose metabolism-related genes. Glucose levels were decreased in HFat diet fed mice compared to Chow diet fed mice. The mRNA levels of glucokinase, the enzyme responsible for the conversion of glucose into glucose-6-phosphate thus enabling the trapping of glucose inside the cells, are increased in HFat diet fed mice (4.32 ± 1.018 –

fold variation to Chow), when compared to both Chow and HFruct diet fed mice (1.00 ± 0.238, and 1.68 ± 0.452 – fold variation to Chow, respectively; Figure 8A). Glucose-6-phosphatase, the enzyme that converts glucose-6-phosphatase to glucose, does not present any significant difference between the groups, although there is a decrease in the HFat group, when compared to Chow (0.45 ± 0.094 and 1.00 ± 0.257 – fold variation to Chow, respectively; p=0.06; Figure 8B). In the fasting state, the liver produces glucose from several substrates, namely glycogen. However, we observed that both HFat and HFruct diet fed mice present lower levels of glycogen in the liver when compared to Chow diet fed mice. Glucose may also enter glycolysis to yield pyruvate; the latter has three major outcomes: acetyl-CoA, acetate or lactate. We observed that hepatic lactate levels do not vary with the diet.

Like for carbohydrates, the liver is essential for fatty acid metabolism. Dysregulations in cholesterol biosynthesis or *de novo* lipogenesis, in a key lipogenic tissue as the liver, may disrupt the overall lipid homeostasis thus, we evaluated intracellular levels of key metabolites for lipid metabolism, as well as the mRNA expression of some lipid metabolism-related genes. *Srebp2* and *Scd1* mRNA levels were increased in HFat diet fed mice (3.06 ± 0.51 and 2.59 ± 0.74 – fold variation to Chow; Figure 8C), when compared to both Chow (1.00 ± 0.29 and 1.00 ± 0.19 – fold variation to Chow) and HFruct groups (1.23 ± 0.26 and 0.76 ± 0.14 – fold variation to Chow; Figure 8C). Glycerol, a product of adipose tissue lipolysis, is an important substrate for both gluconeogenesis and lipogenesis (Patsouris *et al.*, 2004). Glycerol levels were increased in HFat diet fed mice, when compared to Chow diet fed mice. Choline, which is the precursor for the essential component of the VLDL phosphatidylcholine, was increased in HFat diet fed mice, compared to Chow diet fed mice. When glycogen is not available to produce glucose as energy substrate, ketogenesis takes place. One of the metabolites needed for this process is 3-hydroxybutyrate, that was increased in HFat diet fed mice, when compared to Chow and HFruct diet fed animals (Figure 8A).

Amino acids, besides being the basic units for protein synthesis, serve as intermediate metabolites for lipids and nucleotides biosynthesis, as well as cell proliferation and TCA cycle. Amino acid levels were significantly altered in liver tissue by both high-fat and high-fructose diets. Serine, besides being a precursor of proteins and lipids, is involved in glycogen storage in liver. Its levels were decreased in HFat diet fed mice, when compared to both Chow and HFruct diet fed animals(Figure 8A). Serine is inter-convertible with glycine (Alves *et al.*, 2019). The levels of this amino acid were increased in HFat group, when compared to both Chow and HFruct diet fed mice (Figure 8A). During fasting, alanine participates in gluconeogenesis. Alanine levels were increased in HFruct mice when compared to Chow diet fed mice (Figure 8A). α-ketoglutarate (α-KG), one of the metabolites of the Krebs cycle, may be interconverted into glutamate which may be, in turn, interconverted in glutamine. Both glutamate and glutamine were increased in HFat diet fed mice when compared to Chow diet fed animals (Figure 8A). A graphical diagram with the most affected metabolites is depicted in Figure 8A, along with the graphs for each metabolite; and a heat map is depicted in Figure 8D, as well as the most affected pathways in the liver by HFat (Figure 8E) or HFruct feeding (Figure 8F).

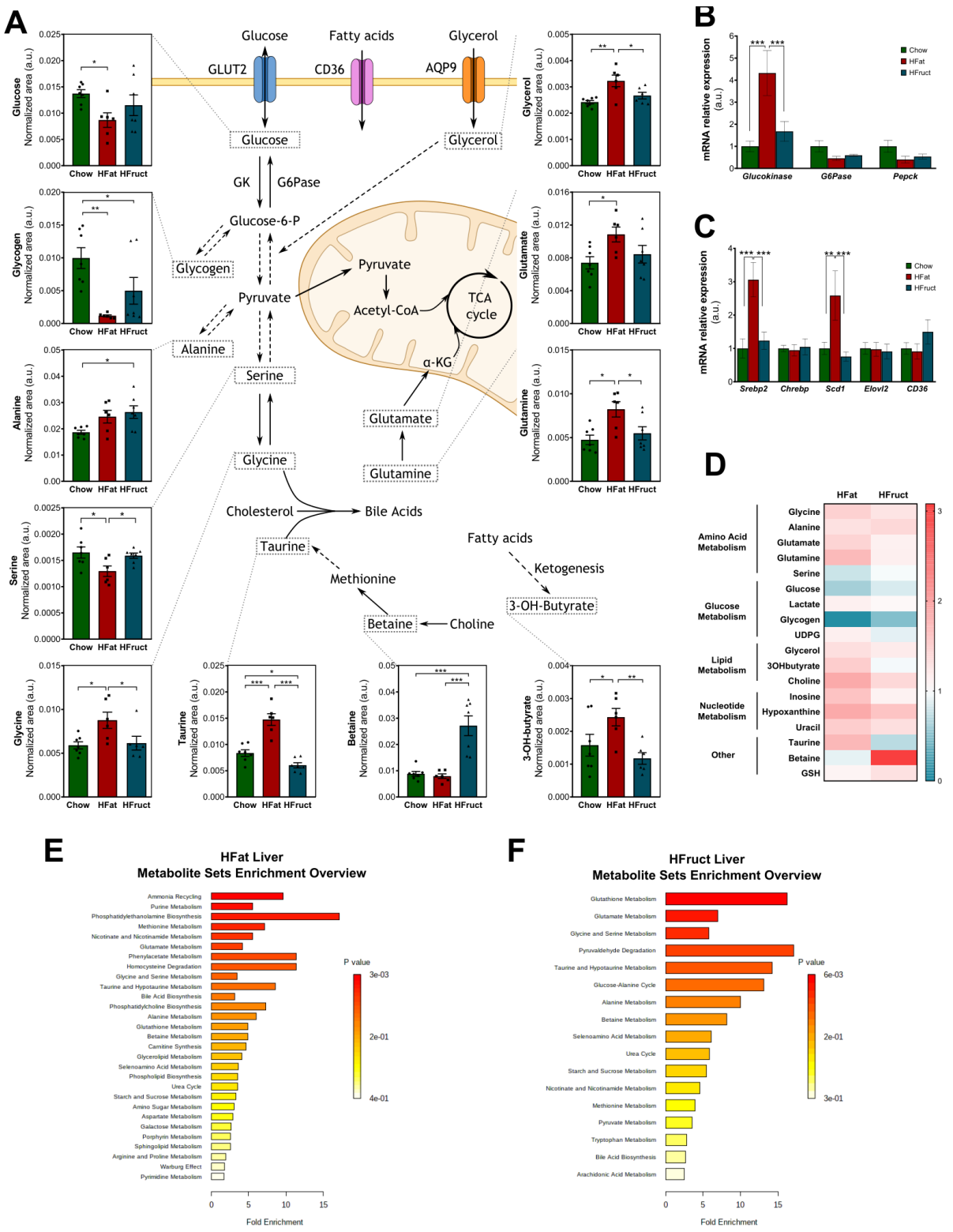

Figure 8 - Effects of the HFat and HFruct diets on the hepatic metabolism of C57Bl/6J male mice. Chow, high-fat (HFat) and high-fructose (HFruct) hepatic metabolites levels (Panel A), mRNA levels of glucose (Panel B) and lipid-related metabolism genes (Panel C), heat map of hepatic polar metabolites in comparison to Chow metabolites, which are represented by 1/white (Panel D) and hepatic metabolite sets enrichment overview of HFat (Panel E) and HFruct mice (Panel F). α-KG – α-ketoglutarate; AQP9 – Aquaporin 9; CD36 – cluster of differentiation 36; G6Pase – Glucose-6-phosphatase; GK – Glucokinase; GSH – glutathione; TCA – Tricarboxylic acid; UDPG - Uridine diphosphate glucose. Data are presented as mean ± SEM for 5-7 mice. * $p<0.05$, ** $p<0.01$; *** $p<0.001$.

High-fat and high-fructose diets differentially affect muscle metabolome

Skeletal muscle is a major organ of glucose uptake, storage, and usage. Muscle can store glucose in the form of glycogen, which is crucial for the rapid initiation of energy production even when glucose is not readily available. Therefore, we evaluated the muscle intracellular metabolites after 12 weeks of HFat or HFruct feeding. A graphical diagram with the most affected metabolites is depicted in Figure 9A, along with the graphs for each metabolite; and a heat map is depicted in Figure 9B, as well as the most affected pathways in muscle by HFat (Figure 9C) or HFruct feeding (Figure 9D). Glucose levels were decreased in HFat diet fed mice, compared with both Chow and HFruct diet fed mice. Lactate, that may be converted into pyruvate, was also decreased in HFat diet fed mice compared with both Chow and HFruct diet fed mice. In the need of substrate, amino acids are metabolized (Moro *et al.*, 2016). In fact, dietary changes had the most impact in amino acids. Glycine and glutamine were increased in HFat diet fed mice compared to the other diets, valine was decreased in both HFat and HFruct diet fed mice when compared to Chow diet fed mice. Both dietary interventions caused a decrease in valine, but only HFruct led to decreased levels of glutamate when compared to Chow diet fed mice.

Brown adipose tissue metabolome is modulated by high-fat or high-fructose feeding

Although it has been disregarded for years, studies about the presence of BAT in adults and the discovery of its crosstalk with important metabolic organs, namely the muscle, has brought BAT to the spotlight (Cypess, 2011). Therefore, BAT metabolome was also evaluated. A graphical diagram with the most affected metabolites is depicted in Figure 10A, along with the graphs for each metabolite; and a heat map is depicted in Figure 10B, as well as the most affected pathways in BAT by HFat (Figure 10C) or HFruct feeding (Figure 10D). HFat diet led to a decrease in glutathione (GSH) and glycerol when compared to Chow diet fed mice. On the other hand, HFat caused an increase in acetate, glutamine and acetate compared with Chow diet. HFruct led to an increase of betaine, taurine, glutamine, leucine. On the contrary, led to a decrease in 3-hydroxybutyrate when compared to Chow diet fed mice (Figure 10A).

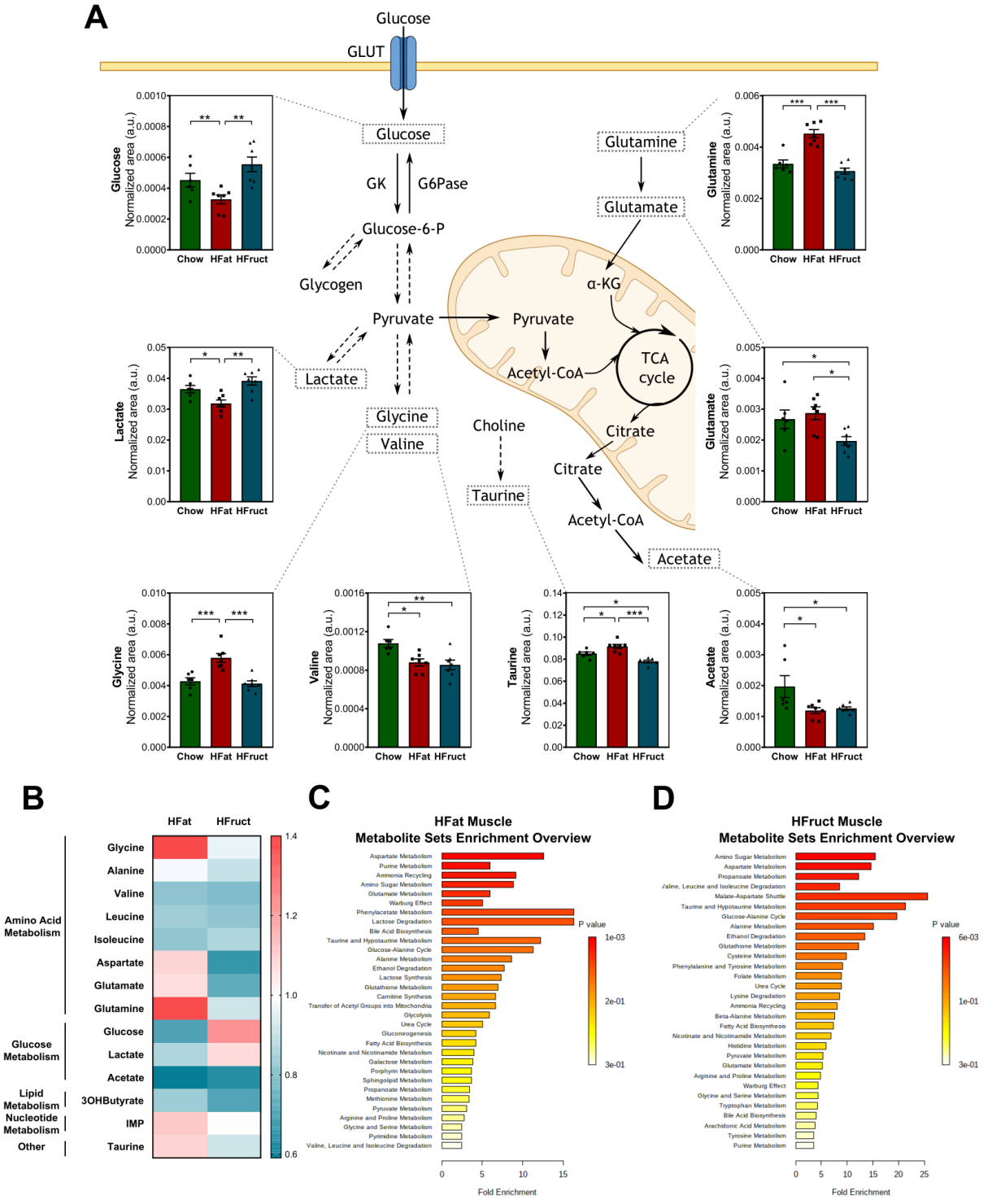

Figure 9 - Effects of the HFat and HFruct diets on the muscle metabolism of C57Bl/6J male mice. Chow, high-fat (HFat) and high-fructose (HFruct) muscle metabolites levels (Panel A), heat map of hepatic polar metabolites in comparison to Chow metabolites, which are represented by 1/white (Panel B) and hepatic metabolite sets enrichment overview of HFat (Panel C) and HFruct mice (Panel D). α-KG – α-ketoglutarate; G6Pase – Glucose-6-phosphatase; GK – Glucokinase; GLUT – Glucose transporter; IMP - Inosine monophosphate; TCA – Tricarboxylic acid. Data are presented as mean ± SEM for 5-7 mice. * $p<0.05$, ** $p<0.01$; *** $p<0.001$.

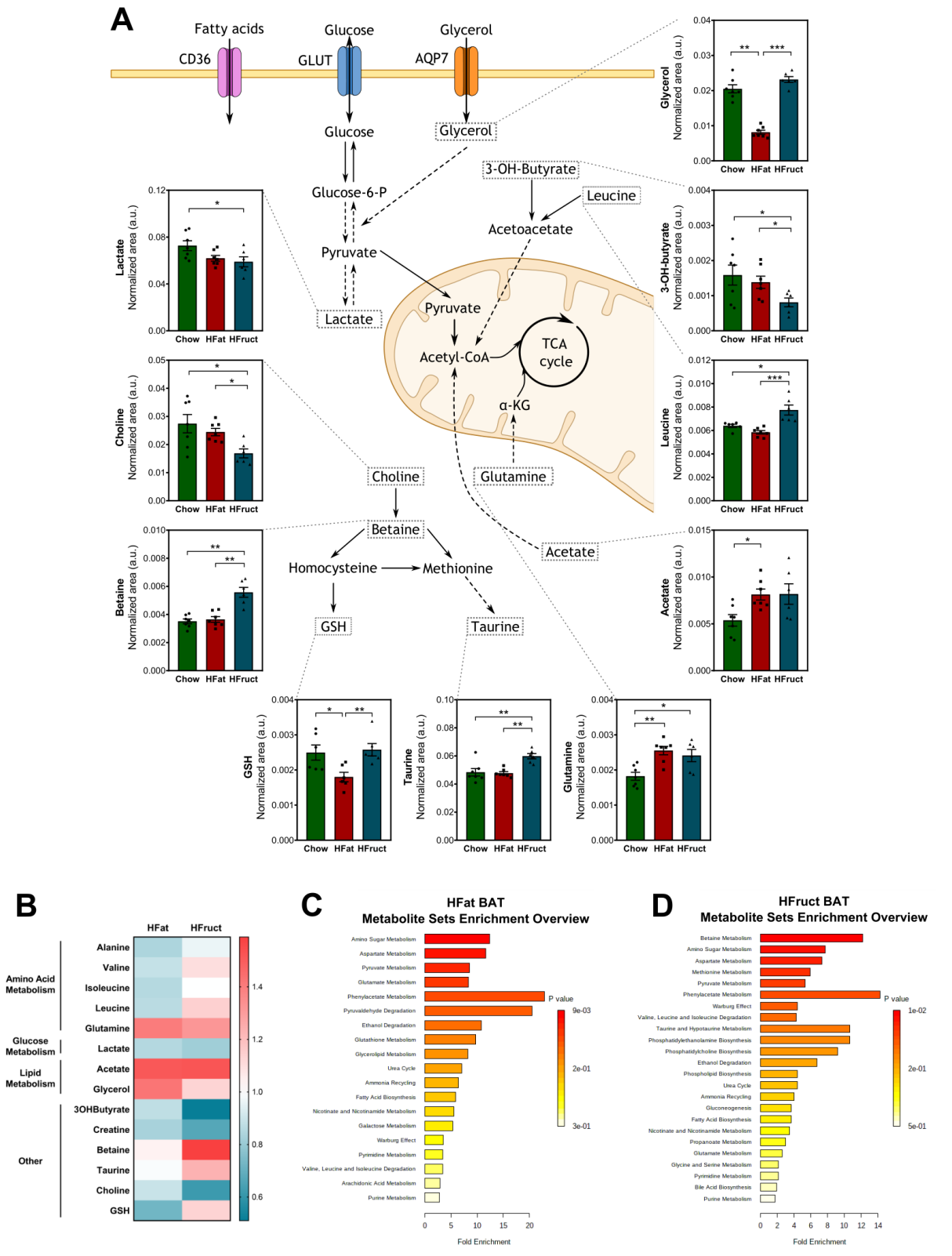

Figure 10 – Effects of the HFat and HFruct diets on the brown adipose tissue (BAT) metabolism of C57Bl/6J male mice. Chow, high-fat (HFat) and high-fructose (HFruct) BAT metabolites levels (Panel A), heat map of hepatic polar metabolites in comparison to Chow metabolites, which are represented by 1/white (Panel B) and hepatic metabolite sets enrichment overview of HFat (Panel C) and HFruct mice (Panel D). α-KG – α-ketoglutarate; AQP7 – Aquaporin 7; CD36 - cluster of differentiation 36; G6Pase – Glucose-6-phosphatase; GK – Glucokinase; GLUT – Glucose transporter; GSH – Glutathione; IMP - Inosine monophosphate; TCA – Tricarboxylic acid. Data are presented as mean ± SEM for 5-7 mice. * p<0.05, ** p<0.01; *** p<0.001.

Hepatic PON1 levels are decreased by both high-fat and high-fructose diets

The overload of lipids in the liver leads to the release of proinflammatory signals by hepatocytes that activate pathways that, when prolonged over time, lead to chronic injury. Endogenous anti-inflammatory proteins, as is the case of PON1, may counteract these inflammatory signals. Considering that PON1 is produced in the liver, the hepatic levels of PON1 were measured. In fact, there was a decrease of PON1 protein levels in both HFat and HFruct diet fed mice (0.56 ± 0.05 and 0.73 ± 0.04 – fold variation to Chow, respectively), when compared to Chow diet fed animals (1.00 ± 0.06; Figure 11). These findings indicate that dietary burden impacts on the hepatic antioxidant machinery, with possible impacts on overall inflammation.

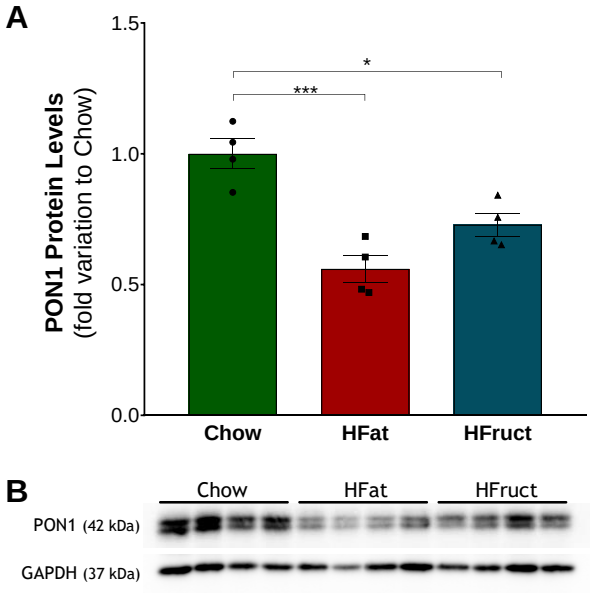

Figure 11 - Hepatic paraoxonase-1 (PON1) protein levels upon 12 weeks of high-fat and high-fructose feeding. A – Chow, high-fat (HFat) and high-fructose (HFruct) hepatic PON1 protein levels normalized by GAPDH protein levels and fold variation to Chow. B – Representative images of hepatic PON1 and GAPDH western blot. Data are presented as mean ± SEM for 4 mice. * $p<0.05$, *** $p<0.001$.

Discussion

The sedentary lifestyle and poor nutritional habits have led to an increase prevalence of metabolic disorders, as obesity, T2DM and NAFLD. Importantly, the increased consumption of highly processed food resulted in increased intake of simple carbohydrates and saturated fat (Rippe and Angelopoulos, 2013). Although it is already known that the enrichment in lipids and in carbohydrates, particularly fructose, has different pathophysiological effects, the consequence of these specific components of the diet on the metabolome of important metabolic organs as the liver, muscle and BAT is not fully understood. Moreover, the impact that these diets have in PON1, an important antioxidant protein, remains to be unveiled.

HFat diet, alone or with sucrose as is the case of the present study, is known to lead to obesity and insulin resistance in mice, originating a phenotype similar to what is observed in humans (Small *et al.*, 2018, Surwit *et al.*, 1995, Wang *et al.*, 2011). On the other hand, fructose alone does not cause a significant increase in body weight (Bergheim *et al.*, 2008). This effect is also seen in humans, and in comparison with glucose, fructose has positive effects regarding body weight (Sievenpiper *et al.*, 2014), despite the deleterious effects in inflammatory processes (Jegatheesan and De Bandt, 2017).

The current study shows that, at 18 weeks of age, HFat diet fed mice had higher caloric intake, body weight and hyperglycemia than Chow diet fed mice. Moreover, HFat diet fed mice were glucose intolerant and insulin resistant. HFruct diet fed mice, on the contrary, presented lower body weight gain than the Chow group and normal glucose tolerance. Although these animals had a decreased food intake, compared to the Chow group, the caloric intake was similar. These results are supported by previous studies, although with a slightly different percentage of fructose (Bergheim *et al.*, 2008, Lindqvist *et al.*, 2008).

The liver is particularly affected by ectopic fat accumulation, as a consequence of an unhealthy diet (Bosy-Westphal *et al.*, 2019, Parry and Hodson, 2017). Though this is already described (Jahn *et al.*, 2019, Yang *et al.*, 2019), this analysis was made in the present study to proper characterize the animal model. HFat mice developed NAFLD, with increased triglycerides and cholesterol. HFruct diet fed mice developed mild hepatic degeneration mainly in periportal areas. This impact of a HFruct diet on liver histology may be due to the activation of a lipogenic pathway and to the absence of a regulatory mechanism of fructose metabolism, contrarily to what happens with glucose (Herman and Samuel, 2016, Softic *et al.*, 2017). In fact, this effect was observed even with only 10% of fructose in drinking water (Yang *et al.*, 2019). A proper glucose usage depends on a functional insulin signaling pathway. However, glucose intolerance and lipid accumulation point towards an impairment of this pathway. Indeed, hepatic insulin signaling was severely affected by both diets. A profound decrease in Akt phosphorylation at both phosphorylation sites was found, demonstrating that these animals have insulin resistance due to an impairment of the insulin signaling pathway upstream of Akt, but downstream InsR (Guo *et al.*, 2009). These results are supported by previous studies where insulin signaling is severely affected due to fructose intake, namely by a decrease in Akt phosphorylation (Softic *et al.*, 2017, Sumlu *et al.*, 2020).

The liver is the most important metabolic organ, regulating carbohydrate, lipid, and protein metabolism (Rui, 2014). While in the fed state, liver uptakes glucose that is either stored as glycogen or converted into fatty acids; in the fasted state, liver produces and releases glucose through glycogenolysis and gluconeogenesis (Rui, 2014). Subsequently, substrates as glucose and triglycerides go into the bloodstream and are metabolized by peripheral organs. Adipose tissue, in turn, releases non-esterified fatty acids and glycerol. Together with alanine and lactate, which are released by the muscle, these metabolites are used as precursors for gluconeogenesis. Hepatic glycogen levels were decreased in HFat diet fed mice. Interestingly, glucokinase expression levels were increased in the liver, while G6Pase levels were unchanged. This might indicate a dysregulation, as in the fasted state it is expected that glucokinase transcription decreases in favor of *G6Pase* expression. Also, in glycolysis, glycerate-3-phosphate can be converted in serine, that is interconvertible with glycine. While serine was decreased in the liver of HFat diet fed mice, glycine was increased. In fact, glycine is involved in the enterohepatic cycle of bile acids, key players in lipid absorption and regulation of cholesterol homeostasis (Alves *et al.*, 2019), that were also increased in this group. Besides glycine, taurine, that is also conjugated with cholesterol for the synthesis of bile acids, was also increased in the liver of HFat diet fed mice, pointing towards an increased production of bile acids. In fact, bile acids are crucial for an effective digestion of lipids (McGlone and Bloom, 2019). As these amino acids and cholesterol were increased only in HFat diet fed mice, it suggests a possible response to the increased lipid intake. Glycerol was increased in the liver of HFat diet fed mice, pointing towards an increase in adipose tissue lipolysis which goes in line with previous studies in adipocytes (Rotondo *et al.*, 2017). Besides, we also observe an increase in hepatic *Srebp2* and *Scd1* expression, suggesting that the lipogenic pathway may be active through a mechanism that may be due to increasing fatty acid levels. On the other hand, the HFruct group also presents a decrease in hepatic glycogen levels, without changes in any of glucose and lipid metabolism-related genes. However, an increase in alanine levels was found, in concurrence with the data obtained in a recent study with HFruct-fed rats (Morrell *et al.*, 2020). This increase in alanine levels may be due to an increase of alanine release from the muscle that is then delivered to the liver through Cahill cycle, or to its conversion from pyruvate in the liver.

The skeletal muscle is a major site for glucose metabolism. Insulin resistance in the muscle is known to cause lipid accumulation, that impairs the tissue's normal functions, ultimately leading to sarcopenia (Rachek, 2014). Muscle stores glucose in the form of glycogen, which facilitates the rapid initiation of energy production for contraction, even when glucose is not readily available from circulation (Rachek, 2014). In this study, HFat diet promoted a decrease in glucose and lactate pools in the muscle, pointing towards the use of other substrates to obtain energy. In the need for other substrates, amino acids may be metabolized. In fact, glutamine levels were increased, and glutamate levels remained similar to the levels detected in the muscle of Chow diet fed mice. Glutamate levels can be used to produce glutamine that will be then delivered to the liver (Cruzat *et al.*, 2018). On the contrary, HFruct diet promoted a decrease in muscular glutamate levels, while glutamine levels were comparable to Chow diet fed mice. Moreover, a decrease in valine was observed in both HFat and HFruct fed groups. Valine is used to obtain energy with the resulting ammonia by-products

participating in the conversion of glutamate to glutamine, thus sustaining the previously mentioned hypothesis. Although it has already been described that diet-induced obese animals have a decreased in branched-chain amino acids (BCAA) (Newgard, 2012), there are studies describing a positive correlation between valine levels and insulin resistance (Tai *et al.*, 2010).

Organ crosstalk is mediated by signaling factors and supports whole-body metabolic homeostasis. However, in case of disease or metabolic dysfunction, metabolic impairment in one organ will cause dysregulation in others. Although this is known, the interplay and metabolic crosstalk between the different organs remain obscure. In recent years, BAT was rediscovered and very recently the existence of a crosstalk with the liver and the muscle was unveiled (Scheele and Wolfrum, 2019). Contrarily to these organs, in the present study BAT metabolome was more affected by HFruct than HFat feeding. HFat led to a decrease in GSH compared to Chow and HFruct diets. GSH was already reported to be inversely correlated with the activation of thermogenesis in white adipocytes due to its action on forkhead box O1 (Lettieri Barbato *et al.*, 2015). Consequently, decreased GSH levels might be increasing thermogenesis in the BAT of high-fat diet fed mice (Mercer and Trayhurn, 1984). Moreover, HFat diet fed mice had increased levels of glutamine and acetate in the BAT. Acetate upregulates mitochondrial biogenesis, which is the key organelle for thermogenesis (Hu *et al.*, 2016). On the other hand, HFruct feeding led to an increase in betaine and taurine, probably at the expense of decreased choline. In fact, betaine protects against fructose-induced inflammation in astrocytes (Li *et al.*, 2015). HFruct diet fed animals also presented increased leucine. This amino acid is an activator of the mammalian target of rapamycin (mTOR) pathway, leading to reduced thermogenesis (Cheng *et al.*, 2010, Wang *et al.*, 2016). Thus, high-fat feeding seems to be an activator of thermogenesis, while HFruct leads to a decrease in this process.

PCA and PLS-DA loadings demonstrate that there is a clearer separation between HFat and Chow diet fed mice than HFruct and Chow fed animals, demonstrating that HFat caused more severe and profound metabolic alterations in the liver, muscle and BAT. In the case of liver and muscle, there was a partial overlap of the metabolic profiles after Chow and HFruct diets, with HFat diet being responsible for a clearer separation from the other two groups. In the BAT, HFat and HFruct metabolome profile was more similar between them and more distinct to Chow. This indicates that HFruct has more impact in BAT, while HFat similarly affects the three organs (Figure 12).

The dysregulation of glucose and lipid homeostasis, due to carbohydrate and lipid overload, leads to inflammation and oxidative stress (Esser *et al.*, 2014). Nevertheless, there are several endogenous intervenients and mechanisms to counteract these deleterious effects (Ali *et al.*, 2020). One of these players is PON1, an important endogenous antioxidant and anti-inflammatory enzyme mainly secreted by the liver (Meneses *et al.*, 2019). Hepatic PON1 levels were affected by both high-fat and high-fructose diets. In fact, HFat decreased hepatic PON1 levels approximately 50% and HFruct induced a reduction about 25%. A recent study with mice fed a high-fat diet with sucrose observed a decrease of 50% in serum arylesterase activity of PON1 (Mahmoudi *et al.*, 2019). Moreover, in rats fed with high-fructose diet a decrease in serum PON1 activity was also observed (Dornas *et al.*, 2012). Therefore, this decrease may be due to decreased secretion of PON1 from the liver and not a decrease

in its activity *per se*. Furthermore, we anticipate our animal models to show a further impairment in PON1 enzymatic activity, contributing to the overall homeostatic dysregulation.

Our data provides evidence that, besides affecting glucose and insulin homeostasis, high-fat and high-fructose feeding differentially affect liver, muscle and BAT metabolic profile. Interestingly, our data suggests a crosstalk between liver, muscle and BAT, particularly regarding alanine, glutamine and lactate. Moreover, hepatic PON1 levels are severely impacted by changes in diet and, particularly, by an increase in lipid and fructose consumption. As PON1 is mainly produced in the liver and only then secreted into the circulation, this decrease may further impact on the organism antioxidant capacity.

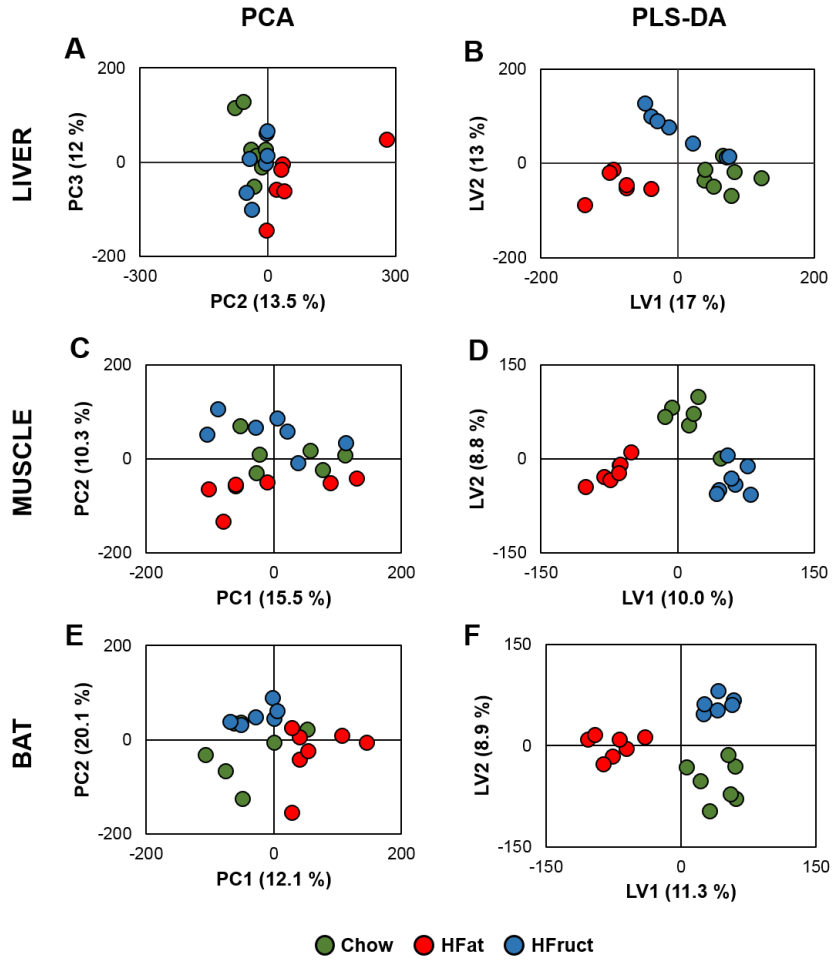

Figure 12 - Multivariate analysis of liver, muscle and brown adipose tissue polar metabolites. A – Liver principal component analysis (PCA) scatter scores plot of Chow, high-fat (HFat) and high-fructose (HFruct) groups; B – Liver partial least square-determinant analysis (PLS-DA) scatter scores plot of Chow, HFat and HFruct groups (permutation test; n=100; R^2=0.94, Q^2=-0.15); C – Muscle PCA scatter scores plot of Chow, HFat and HFruct groups; D – Muscle PLS-DA scatter scores plot of Chow, HFat and HFruct diets (permutation test; n=100; R^2=0.76, Q^2=-0.066); E – Brown adipose tissue (BAT) PCA scatter scores plot of Chow, HFat and HFruct diets; F – BAT PLS-DA scatter scores plot of Chow, HFat and HFruct diets (permutation test; n=100; R^2=0.982, Q^2=0.115).

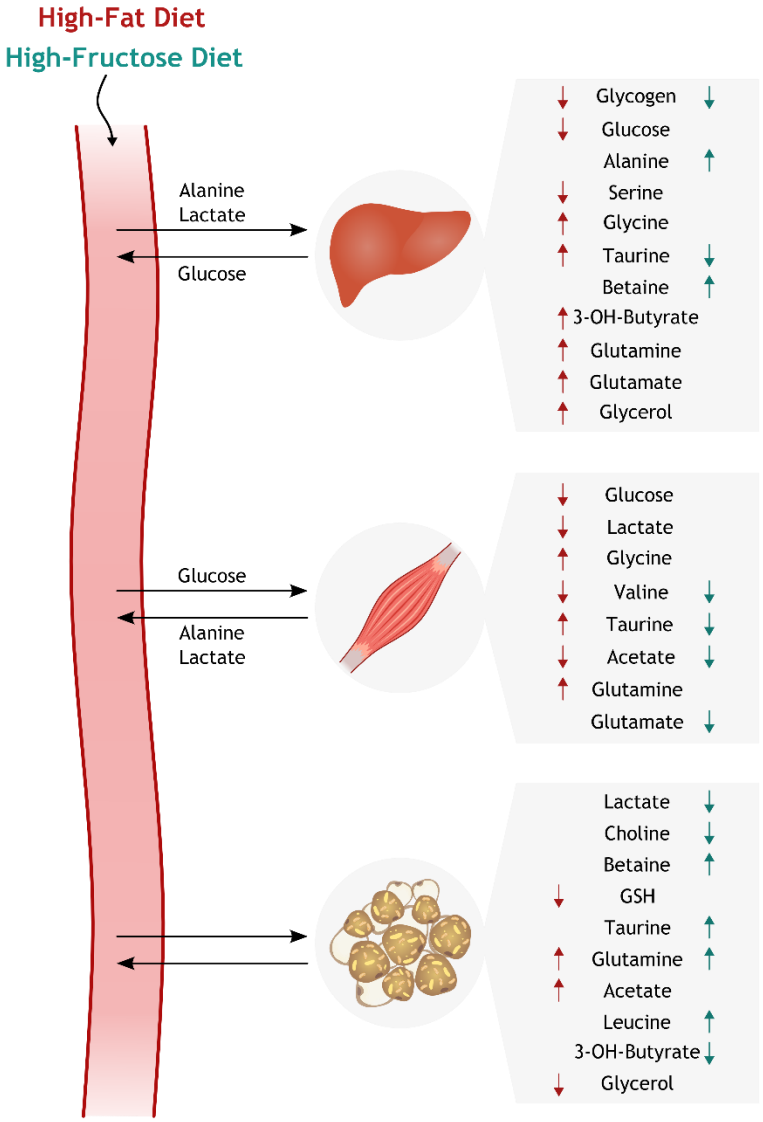

Figure 13 – Summary of the metabolomic analysis in the liver, muscle and brown adipose tissue (BAT) upon high-fat and high-fructose diets and crosstalk between these tissues. ↓ decrease; ↑ increase; high-fat diet; high-fructose diet; 3-OH-butyrate – 3-hydroxybutyrate; GSH - glutathione.

The effects of PON1 deletion and diet insults on hepatic glucose and lipid metabolism

Abstract

The prevalence of metabolic disorders such as obesity, type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD) is increasing exponentially. This increase strongly correlates with the consumption of saturated fat and carbohydrates, which are known to promote a proinflammatory and prooxidant milieu. Paraoxonase-1 (PON1), a liver-expressed protein, may counteract these pathophysiological states due to its antioxidant properties. In this work, we hypothesize that PON1 deletion impacts hepatic metabolism and overall insulin resistance upon high-fat or high-fructose feeding.

Whole-body *Pon1* knockout (KO) and wild-type (WT) mice were given *ad libitum* access to a control (NCD), high-fat (HFat) or high-fructose diet (HFruct) from 6 to 18 weeks. Mice were monitored for body weight and blood glucose levels. Whole-body homeostasis was determined through an insulin tolerance test (ITT) and a glucose tolerance test (GTT). Insulin, C-peptide, triglycerides and cholesterol levels were assessed. Liver sections were stained, and their lipid content was determined. The hepatic insulin signaling pathway activation was studied, as well as the expression of lipid and glucose metabolism related genes.

NCD KO and HFD KO mice were more glucose intolerant than the WT although body weight gain, fasting blood glucose levels and insulin sensitivity were unchanged between the groups. C-peptide levels were increased in KO, while insulin levels remained unchanged. Cholesterol and triglycerides levels were unchanged, both in serum and hepatic tissue, and no changes were observed regarding hepatic fat deposition. Akt phosphorylation is increased in NCD and HFruct KO mice.

This study provides evidence that the absence of PON1 has a relevant impact on glucose homeostasis, independently of obesity and ectopic fat deposition.

Keywords: Paraoxonase-1; High-fat; Fructose; Diet; Liver; Hepatic metabolism; Glucose intolerance.

Introduction

Paraoxonase-1 (PON1) is a Ca^{2+}-dependent enzyme with a molecular weight of approximately 43 kDa and 354 amino acids (She *et al.*, 2012). This enzyme was found due to the ability to hydrolyze paraoxon, a metabolite of the insecticide parathion. However, as paraoxon is not a natural compound, it could not be the physiological substrate of PON1 (Mackness and Mackness, 2015). The latter is now known to have a wide variety of substrates, namely lactones, thiolactones and aryl esters (Davies *et al.*, 1996). PON1 is mainly produced in the liver and, in circulation, it is mainly found bound to HDL. In fact, the atheroprotective effect of HDL, due to its capacity of preventing LDL oxidation, is now attributable to PON1 (Mackness and Mackness, 2010). Besides, variations in circulating PON1 levels were also associated with a variety of diseases involving oxidative stress and inflammation, as is the case of metabolic disorders (Gupta *et al.*, 2011). In fact, hepatic steatosis, the first feature of non-alcoholic fatty liver disease (NAFLD), is the most common liver disease in Western societies (Bellentani, 2017). Although it might also have a genetic contribution (Romeo *et al.*, 2020), one of the main risk factors for the onset of NAFLD is the diet and, specifically, a diet that is rich in fructose, sucrose and saturated fats. These specific components of the diet lead to increased free fatty acid levels in the hepatocytes, a result of increased incursion of FFAs from the insulin-resistant adipose tissue, impaired processing of dietary lipids in the liver or lipid export from the hepatocytes and increased hepatic *de novo* lipogenesis (Bosserhoff and Hellerbrand, 2011). This excessive amount of FFAs may lead to the formation of lipotoxic species, that will then result in inflammation and oxidative stress (Friedman *et al.*, 2018).

Due to the anti-inflammatory and antioxidant actions of PON1, we hypothesize that PON1 deletion changes hepatic metabolism and overall insulin resistance. The aim of this study was to determine the effects of PON1 deletion, namely on glucose homeostasis and in the liver of mice fed with a control diet or with a further insult of a western or a high-fructose diet.

Material and Methods

Chemicals

BCA Protein Assay Kit was purchased from Thermo Scientific (Whalthan, MA, USA). Dried milk was purchased from Nestlé. Amersham ECL was purchased from GE Healthcare (Weßling, Germany). NZYColour Protein Marker II, agarose, NZYDNA Ladder V, Supreme NZYTaq II 2x Green Master Mix, and Proteinase K were purchased from NZYTech (Lisbon, Portugal). Fructose was purchased from Enzymatic (Loures, Portugal). Immobilon-P PVDF Membrane was purchased from Merck Millipore (Massachusetts, USA). Triglycerides and Cholesterol kits were purchased from Spinreact (Girona, Spain). Free fatty acids kit was purchased from Wako Diagnostics (CA, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless stated otherwise.

Mice generation, genotyping and experimental design

B6.129X1-*Pon1*^{tm1Lus}/J were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Heterozygous breeding generated whole-body *Pon1* knockout (KO), heterozygous (Het) and wildtype (WT) littermates (Figure 14). Mice genotyping was performed using DNA isolated from the tail. Mice were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. The experimental procedures received prior approval by the Ethics Committee of the NOVA Medical School (82/2019/CEFCM and 02/2020/CEFCM) and by the Directorate-General for Food and Veterinary that regulates the animal care and use in research. All procedures followed ARRIVE guidelines and the Europeans laws (Directive 2010/63/EU) that rule the use of animals in research. At 6 weeks of age, mice were randomly divided in 3 groups to be fed with a normal chow diet (NCD; RM3A (P), Special Diets Services, Witham, Essex, UK); a high-fat diet with 58% kcal in fat (HFat; D12331, Research Diets, New Brunswick, NJ) or a high fructose diet (HFruct; FRUC-00T-500; 35% w/v in drinking water).

Metabolic Measurements

Mice were weighed after an overnight fasting period at 6 weeks of age and every two weeks thereafter. At the same time, blood was also collected, and blood glucose levels were measured using a Contour Next glucose meter (Bayer, Leverkusen, Germany). Serum insulin and C-peptide were measured by enzyme-linked immunosorbent assay (Crystal Chem Inc., Chicago, IL). Insulin clearance levels were measured as the ratio between C-peptide and insulin. Serum free fatty acids (Wako Diagnostic), total cholesterol and triglyceride levels were determined by enzymatic method (Spinreact, Girona, Spain) following manufacturers' intructions. For glucose tolerance tests, mice were fasted overnight at 17 weeks of age, and blood glucose was measured before and 15, 30, 60 and 120 min after intraperitoneal injection of glucose (2.0 g/kg of body weight). For insulin tolerance tests, mice were fasted for 5 h at 17 weeks of age, and blood glucose was measured before and 15, 30, 60 and 120 min after intraperitoneal injection of human insulin (0.5 UI/kg of body weight; Actrapid, Novo Nordisk). Area under the curve was calculated using the trapezoidal rule for glucose or insulin data (AJP).

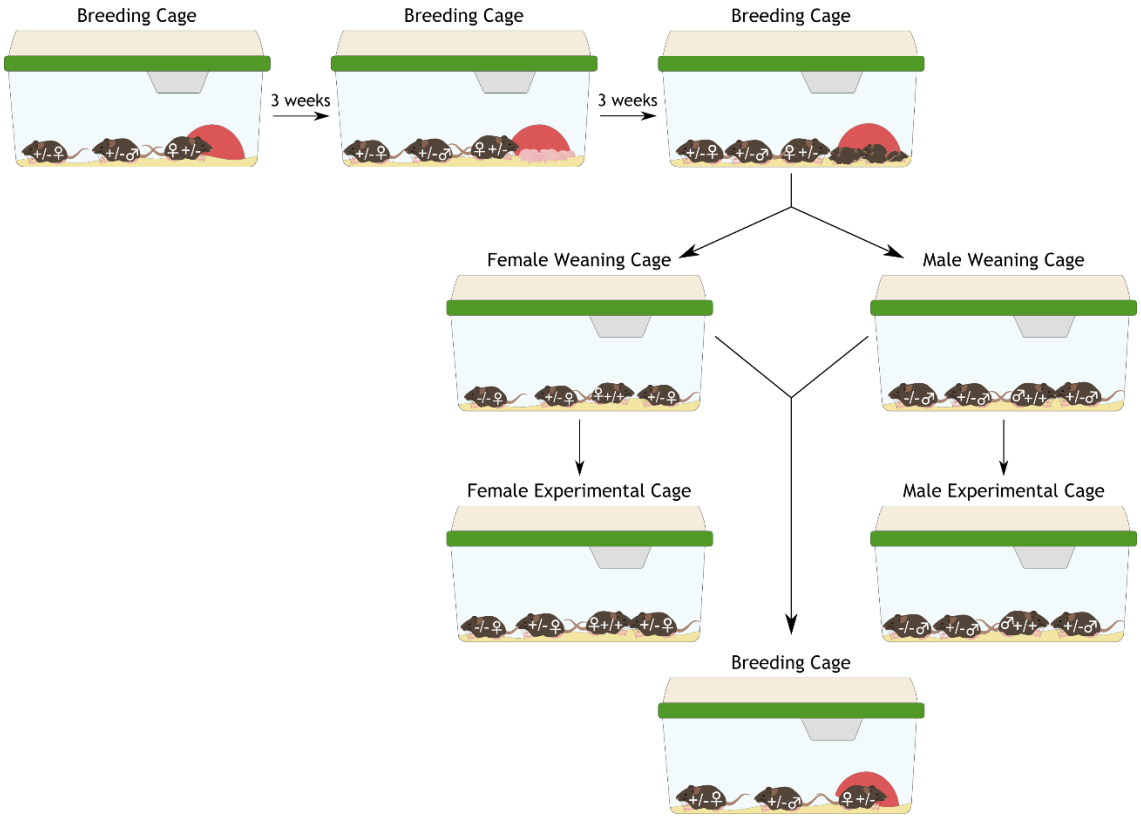

Figure 14 – Breeding Scheme. In this study, breeding cages were composed of one male and two females. To get both wildtype (+/+) and KO (-/-) littermates, the parents were heterozygous (+/-) for *Pon1*. It takes around 3 weeks for mice to gestate and another 3 weeks for the litter to be weaned. After this time, mice are separated by gender and genotyped, knowing that normally the mice will be 25% +/+, 50% +/- and 25% -/-. After genotyping, experimental cages with mixed genotypes and new breeding cages, if necessary, are set up.

Serum Paraoxonase activity

Serum paraoxonase activity was measured as previously described (Batuca *et al.*, 2007). Paraoxon (1 mM; Sigma-Aldrich) was freshly prepared in 290 µL of 50 mM glycine buffer containing 1 mM calcium chloride (pH 10.5). Then, the latter was incubated with 10 µL of serum at 37°C for 40 min in 96-well plates. p-Nitrophenol formation was monitored at 412 nm and activity expressed in U per L of serum.

Insulin signaling studies

Mice were fasted overnight and injected intraperitoneally with human insulin (10 UI/kg; Actrapid) or saline and sacrificed 10 min later. The liver was rapidly harvested, snap frozen and stored at -80°C until further analysis. Liver was homogenized in lysis buffer (in mM: 20 Tris pH 7.5, 5 EDTA, 10 $Na_4P_2O_7$, 100 NaF, 2 Na_3VO_4) with 1% NP-40 and protease inhibitors (Roche, Switzerland). Tissue lysates (15-20 µg protein) were resolved by SDS-PAGE and transferred to PVDF membranes with TransBlot Turbo (Bio-Rad Laboratories, California, USA). The membranes were incubated with the antibodies listed in Table 4 and either GAPDH or ß-actin were used as loading controls. The bands were visualized with Chemidoc (Bio-Rad Laboratories) and quantified using ImageLab (Bio-Rad Laboratories).

Table 4 – List of antibodies used to evaluate the impact of *Pon1* deletion on hepatic insulin signaling pathway.

Antibody	Host Specie	Molecular Weight (kDa)	Dilution	Vendor	Catalog #
InsR-α	Rabbit	95	1:1000	Cell Signaling	74118
pInsR	Rabbit	95	1:1000	ThermoFisher Scientific	44-806G
Akt	Rabbit	60	1:1000	Santa Cruz Biotechnology	sc-7582
pAkt$_{S473}$	Rabbit	60	1:1000	Cell Signaling Technology	4060
pAkt$_{T308}$	Rabbit	60	1:1000	Cell Signaling Technology	4056
PON1	Rabbit	43	1:2000	Abcam	ab126597
GAPDH	Mouse	37	1:10000	Ambion	AM4300
β-Actin	Mouse	42	1:5000	Cell Signaling Technology	3700
Mouse	Goat	—	1:5000	Santa Cruz Biotechnology	sc-2031
Rabbit	Goat	—	1:5000	Santa Cruz Biotechnology	sc-2030

InsR-α – Insulin receptor alpha; pInsR – phosphorylated insulin receptor; Akt – protein kinase B; pAkt – phosphorylated protein kinase B; PON1 – paraoxonase-1; GAPDH - glyceraldehyde-3-phosphate dehydrogenase.

Liver histology and lipid assay

Liver was fixed with 10% formalin solution, embedded in paraffin and stained with hematoxylin-eosin staining for characterization of liver morphology and lipid content. Hepatic lipids were extracted as previously described (Matyash *et al.*, 2008). Briefly, approximately 125 mg of frozen livers were rapidly mixed with HPLC-grade methanol (4.6 mL/g) followed by methyl-tert-butyl ether (MTBE) (15.4 mL/g). The mixture was placed in a shaker for 3 h and then centrifuged at 13,000 *.g* for 10 min. The liquid fraction was collected, and phase separation was induced by adding 600 µL of distilled water and letting it rest at room temperature for 10 min. The liquid was then centrifuged for 10 min at 1000 *.g*. The organic phase which contained the lipids was separated and dried under nitrogen gas in an amber glass vial. It was then dissolved in butanol/(Triton X-100:methanol [2:1]). Hepatic total cholesterol and triglyceride contents were determined by enzymatic method following manufacturers' recommendations (SpinReact).

Quantitative real-time PCR

Liver total RNA was extracted using TRIzol (Invitrogen) and subjected to quantitative real-time PCR as described in the previous chapter. Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) with β-actin and β-2-microglobulin normalization. Gene-specific primer sequences are listed in Table 5.

Table 5 – Primer sequences and cycling conditions for polymerase chain reactions to evaluate the impact of *Pon1* deletion in glucose and lipid-related metabolism gene expression.

Gene	Sequence 5' -3'	Annealing Temperature	Cycles
Glucokinase (NM_010292.5)	Fwd: GCTGGTACGACTTGTGCTG Rvs: TGGACACGCTTTCACAGG	60°C	40
G6Pase (NM_008061.4)	Fwd: ATGAACATTCTCCATGACTTTGGG Rvs: GACAGGGAACTGCTTTATTATAGG	60°C	40
PEPCK (NM_011044.3)	Fwd: TGAAAGGCCGCACCATGTAT Rvs: GCACAGATATGCCCATCCGA	60°C	40
LXR α (NM_001355279.1)	Fwd: AGGAGTGTCGACTTCGCAAA Rvs: CTCTTCTTGCCGCTTCAGTTT	58°C	40
ChREBP (NM_001359237.1)	Fwd: CTGGGGACCTAAACAGGAGC Rvs: GAAGCCACCCTATAGCTCCC	60°C	40
SREBP2 (NM_033218.1)	Fwd: GCGTTCTGGAGACCATGGA Rvs: CACAAGTTGCTCTGAAAACAAATCA	60°C	40
Elovl2 (NM_001311121.1)	Fwd: CCTGCTCTCGATATGGCTGG Rvs: AAGAAGTGTGATTGCGAGGTTAT	60°C	40
CD36 (NM_001159558.1)	Fwd: GATGACGTGGCAAAGAACAG Rvs: TCCTCGGGGTCCTGAGTTA	58°C	40
SCD1 (NM_009127.4)	Fwd: CGCCCCTACGACAAGAACAT Rvs: CTCAGAAGCCCAAAGCTCAG	60°C	40
ß-actin (NM_007393.5)	Fwd: CGTGAAAAGATGACCCAGATCA Rvs: CACAGCCTGGATGGCTACGT	60°C	40
ß-2-microglobulin (NM_009735.3)	Fwd: TCTCACTGACCGGCCTGTAT Rvs: CAGTCTCAGTGGGGGTGAAT	60°C	40

Statistical Analysis

Experimental data are shown as mean ± SEM. Statistical analysis was performed using one or two-way ANOVA in Graphpad Prism 8 (GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered significant.

Results

Generation of Pon1 *KO mice*

In this work, the physiological function of PON1 was assessed by studying whole-body *Pon1* KO mice. Heterozygous breeding generated *Pon1* KO mice, heterozygous animals and control littermates (WT). *Pon1* deletion was confirmed by the absence of the protein in liver lysates (Figure 15A) and by the absence of serum paraoxonase (POase) activity (Figure 15B). The heterozygous animals had intermediate levels of both hepatic PON1 protein levels and POase activity.

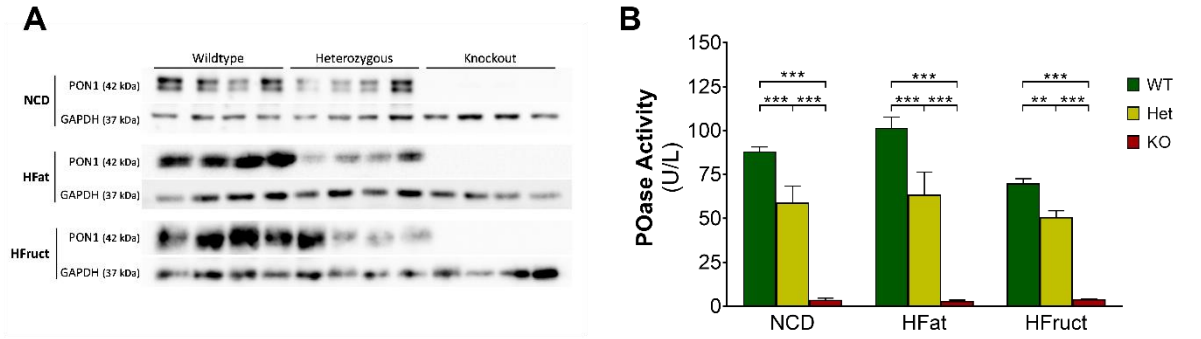

Figure 15 – Hepatic PON1 protein levels and serum paraoxonase activity. Representative images of hepatic PON1 and GAPDH protein levels determined by western blot (panel A) and serum paraoxonase activity (POase, panel B) of wildtype (WT), heterozygous (Het) or knockout (KO) mice for *Pon1* fed with Normal Chow Diet (NCD), High-fat (HFat) or High-fructose (HFruct) diet. Data in Panel B is presented as mean ± SEM for 6-7 mice. ** $p<0.01$; *** $p<0.001$.

Pon1 *deletion exacerbates glucose intolerance upon high-fat diet*

Pon1 deletion did not affect body weight gain in normal chow diet (NCD) fed mice, neither in mice with different dietary regimens, namely high-fat (HFat) or high-fructose (HFruct) diets (Figure 16A-C, respectively). To analyze if *Pon1* deletion had an effect in fat deposition, different fat depots, both white and brown, were weighed and the fat depot / body weight ratio was performed. However, no differences were found (Figure 16D-F). Although insulin tolerance appears to be unchanged upon *Pon1* deletion (Figure 16G-I), *Pon1* KO mice fed an HFat diet present decreased glucose tolerance when compared to WT mice fed with the same diet (Figure 16K). However, when analyzing the AUC of the GTT, which is an index of whole glucose excursion after glucose loading, we verify that, besides HFat KO, NCD KO mice also have glucose intolerance when compared to the NCD WT mice (Figure 16J).

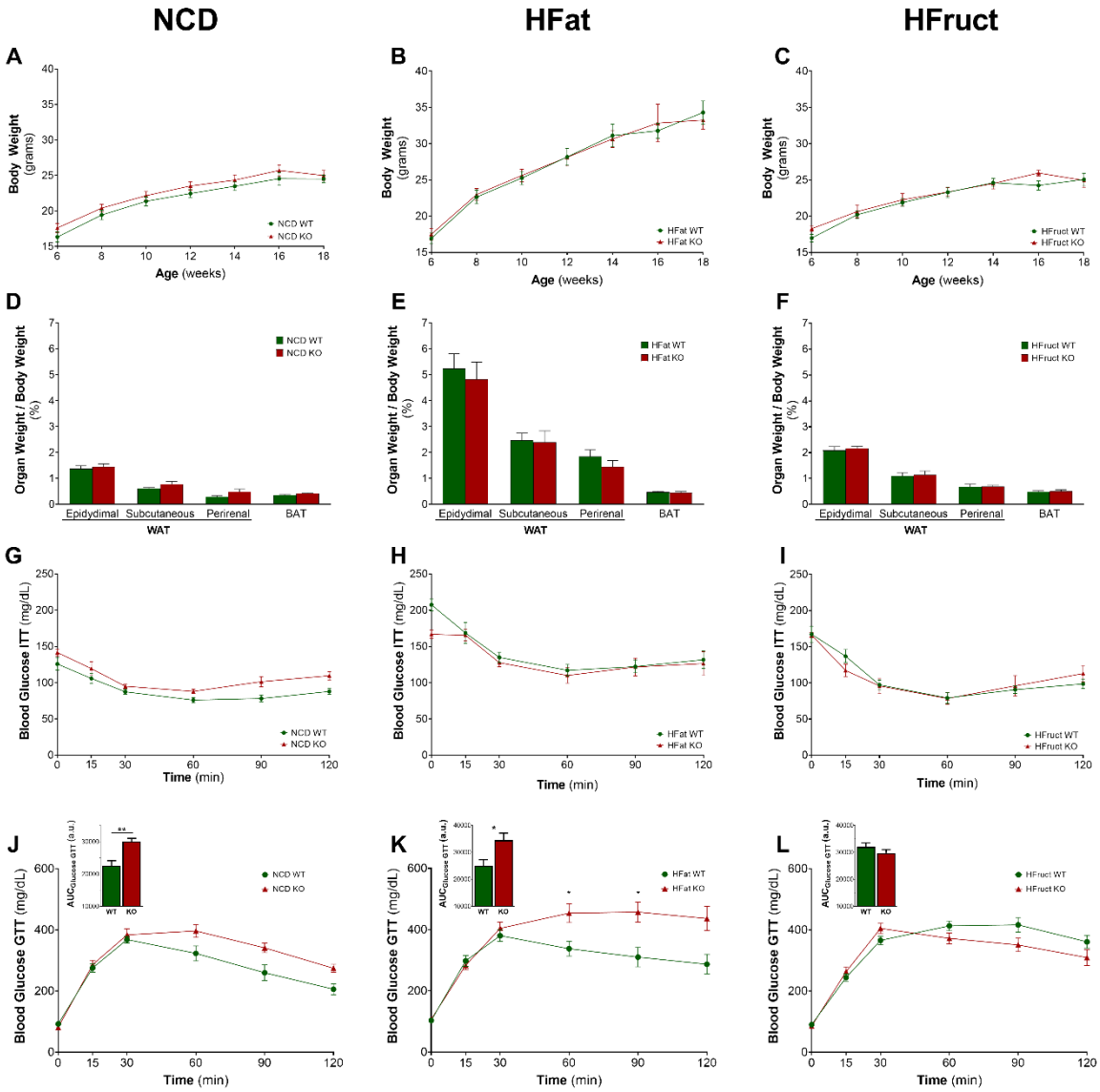

Figure 16 – Effects of *Pon1* deletion under Normal Chow Diet, High-Fat or High-Fructose feeding on body weight gain, fat accumulation and glucose homeostasis. Body weight (Panels A-C), fat depots weight (Panels D-F), insulin tolerance test (Panels G-I) and blood glucose tolerance tests and respective area under the curve (AUC; Panels J-L) of wildtype (WT) and *Pon1* knockout (KO) mice fed normal chow diet (NCD), high-fat diet (HFat) or high-fructose diet (HFruct), respectively. Data are presented as mean ± SEM for 6-10 mice. * p<0.05; ** p<0.01.

Insulin clearance is increased upon Pon1 *deletion*

Under a normal physiological state, up to 70% of secreted insulin is cleared during its first passage through the liver (Duckworth *et al.*, 1998). To assess insulin homeostasis, C-peptide levels, that reflect insulin secretion by the pancreas (Weiss *et al.*, 2000), and insulin levels were measured during the glucose tolerance test. C-peptide levels were increased in NCD KO mice when compared to NCD WT mice (Figure 17A-C), without affecting overall insulin levels (Figure 17D-F). Although there was a similar tendency in HFat or HFruct diet fed mice, it did not reach statistical significance. However, insulin clearance is increased in KO mice when compared to WT mice, regardless of the diet, being clearer in NCD and HFruct mice (Figure 17G-I).

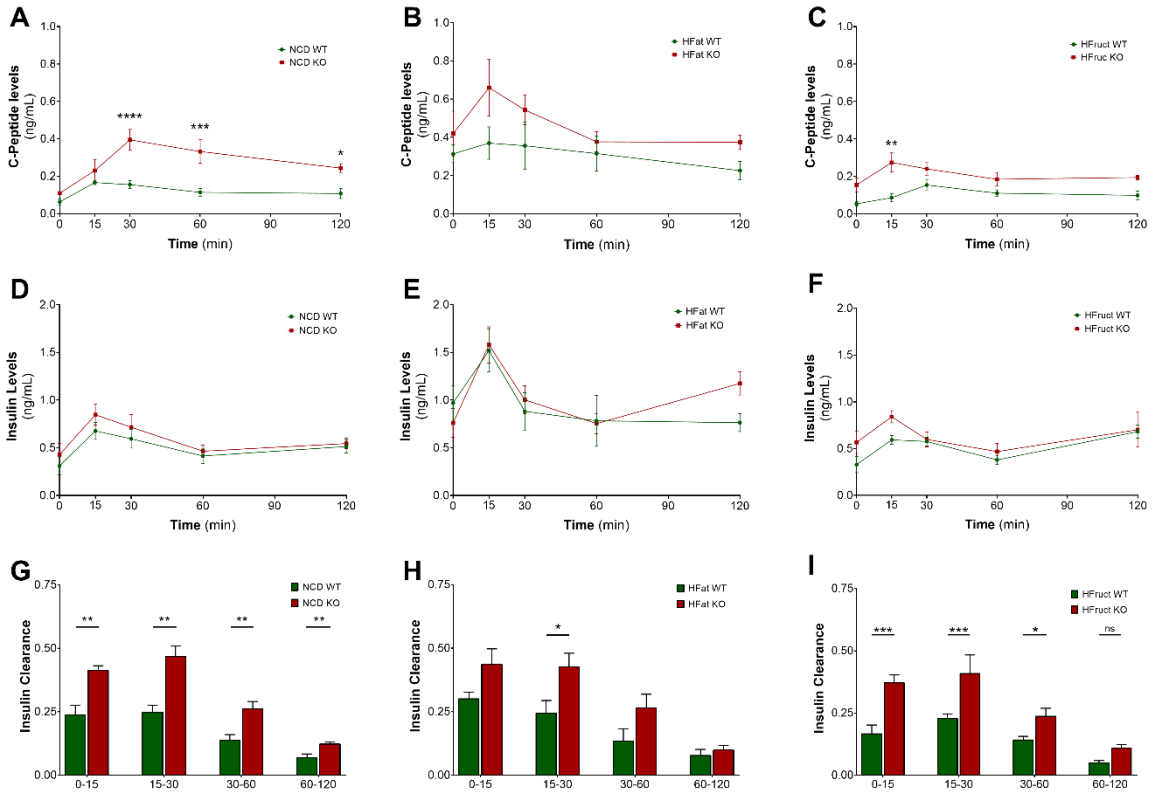

Figure 17 – Effects of *Pon1* deletion under Normal Chow Diet, High-Fat or High-Fructose feeding on insulin homeostasis. C-peptide (Panel A-C), insulin levels (Panel D-F), and insulin clearance (Panel G-I) of wildtype (WT) and *Pon1* knockout (KO) mice fed with normal chow diet (NCD), high-fat diet (HFat) or high-fructose diet (HFruct), respectively. Data are presented as mean ± SEM for 6-7 mice. * p<0.05, ** p<0.01, *** p<0.001.

Pon1 deletion increases hepatic Akt phosphorylation in mice under NCD or HFruct

Insulin-stimulated glucose utilization relies on a fully functional insulin signaling pathway. Therefore, the hepatic protein levels of the insulin receptor (InsR) and protein kinase B (Akt) in the liver of the mice were determined. No differences were found between WT and KO mice regarding both InsR and pInsR levels, in neither of the diets (Figure 18). However, there was a significant increase in pAktT308 in NCD KO mice compared to NCD WT (Figure 18A). Moreover, there was an increase in HFruct KO pAkt protein levels, in both phosphorylation sites, when compared with HFruct WT (Figure 18C). Thus, *Pon1* deletion impacts differently in Akt phosphorylation, depending on the diet.

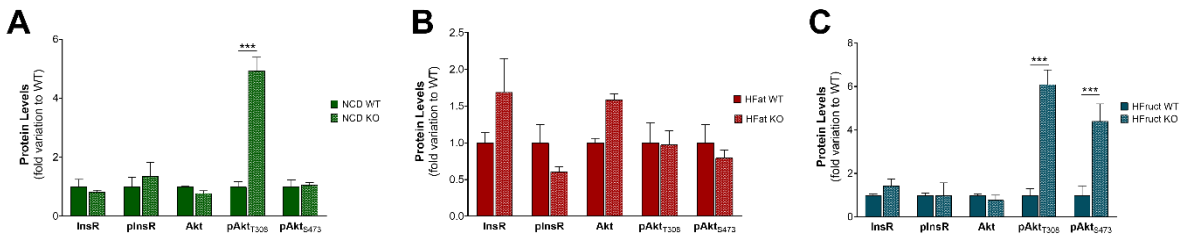

Figure 18 – Effects of *Pon1* deletion under Normal Chow Diet, High-Fat or High-Fructose feeding on hepatic insulin signaling pathway. Protein levels of Insulin Receptor (InsR), phosphorylated InsR (pInsR), protein kinase B (PKB/Akt), and phosphorylated Akt (either on Thr308 or Ser473) in wildtype (WT) of *Pon1* knockout (KO) mice fed with normal chow diet (NCD; Panel A), high-fat diet (HFat; Panel B) or High-fructose diet (HFruct; Panel C). Data are presented as mean ± SEM for 3-4 mice. *** p<0.001.

Pon1 *deletion does not affect serum triglycerides nor cholesterol*

To evaluate the effects of PON1 deficiency on lipid profile, serum levels of free fatty acids, triglycerides, and cholesterol were measured. No differences were found in any of the three diets between the WT and the KO groups (Figure 19A-C). However, and as expected, both serum triglycerides and cholesterol were increased in HFat diet fed mice, when compared to NCD and HFruct diet fed animals (Figure 19B-C).

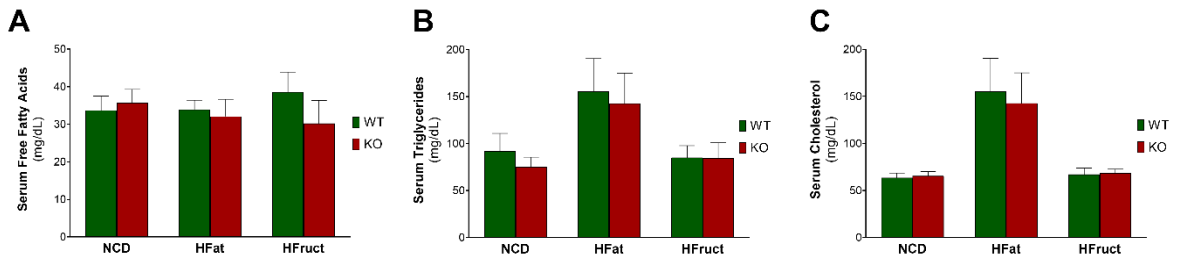

Figure 19 – Effects of *Pon1* deletion under Normal Chow Diet, High-Fat or High-Fructose feeding on serum lipid profile. Free fatty acids (Panel A), triglycerides (Panel D-F), and cholesterol (Panel G-I) levels in the serum of wildtype (WT) and *Pon1* knockout (KO) mice fed with normal chow diet (NCD), high-fat diet (HFat) or high-fructose diet (HFruct). Data are presented as mean ± SEM for 6-7 mice.

Pon1 *deletion does not affect hepatic fat deposition*

To investigate the effects of *Pon1* deletion on hepatic lipid accumulation, we measured hepatic triglyceride and cholesterol contents in the liver of the mice under NCD, HFat or HFruct diets, as well as the mRNA expression of genes involved in lipid metabolism. No differences were found regarding the liver/body weight ratio (Figure 20A). The analysis of both liver triglycerides and cholesterol showed no differences due to *Pon1* deletion (Figure 20B-C), that were further verified by the pathological analysis of liver histological sections (Figure 20D). Moreover, the expression of lipid metabolism-related genes was assessed after an overnight fasting (Figure 20E). The expression of *Cd36*, responsible for the uptake of free fatty acids, was increased in HFruct diet fed mice, both WT and KO, independently of *Pon1* deletion. Regarding genes related with *de novo* lipogenesis, that are expected to be downregulated in fasting, *Pon1* deletion caused no changes in the expression of *Acc*, *Fas*, *Elovl2* and *Srebp2*. However, *Acc* expression was decreased in HFat and HFruct diet fed mice compared to NCD, independently of the presence of PON1. Moreover, HFat diet led to an increase of *Scd1* expression, the gene coding for the enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids, when compared to NCD WT. *Pon1* deletion in HFat diet fed mice restored *Scd1* expression to levels similar to NCD WT. Besides, the expression of *Chrebp*, that regulates gene transcription related to both glucose and lipid metabolism, was increased in NCD KO mice compared with NCD WT. On the other hand, the absence of PON1 upon HFruct decreased *Chrebp* expression compared with HFruct WT mice.

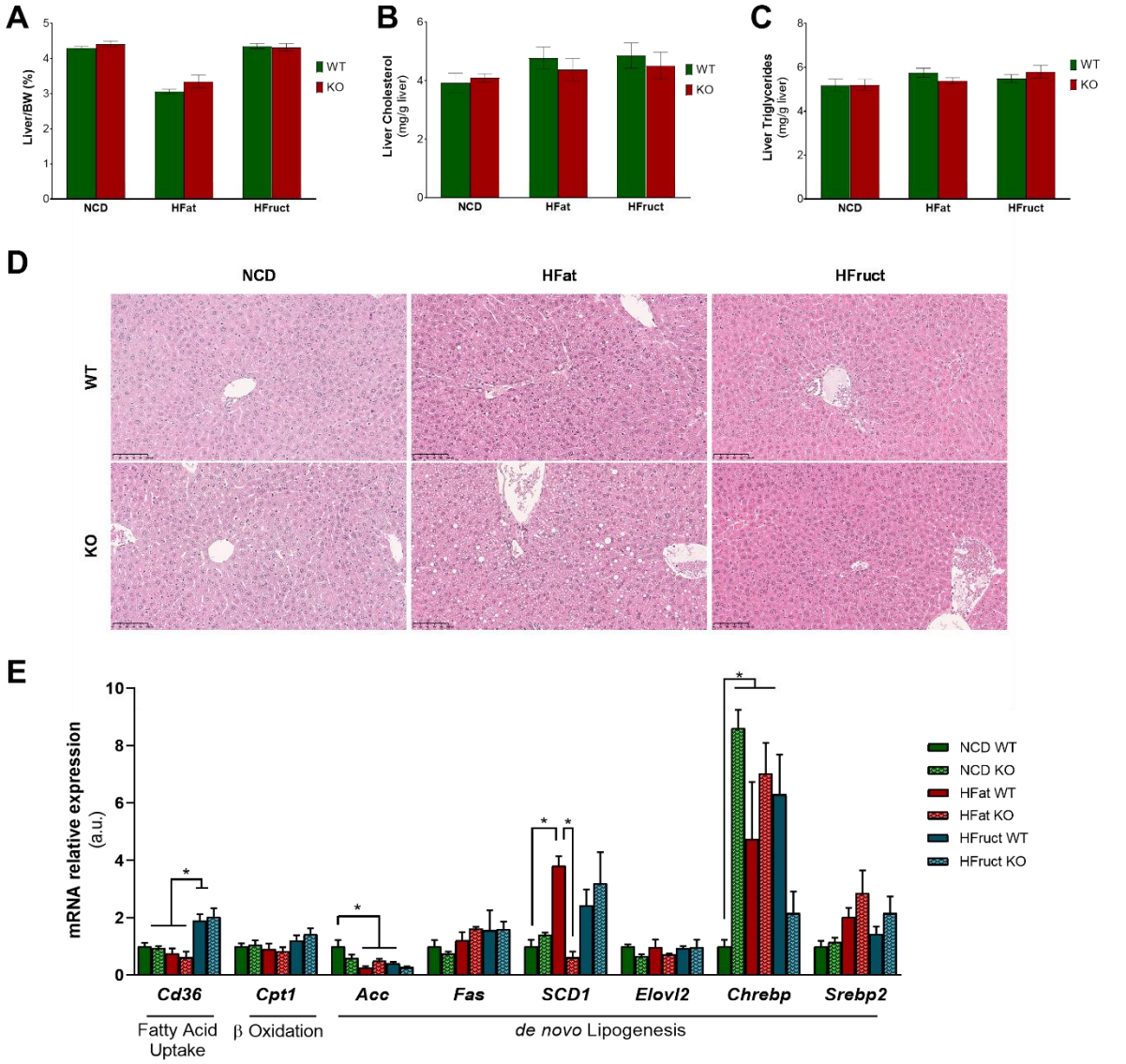

Figure 20 – Effects of *Pon1* deletion under Normal Chow Diet, High-Fat or High-Fructose feeding on liver lipid content and mRNA expression of lipid metabolism-related genes. Panel A - Liver / body weight (BW) ratio; Panel B - Liver triglycerides; Panel C - Liver cholesterol; Panel D – Representative images of hematoxylin & eosin-stained liver sections; and Panel E - Gene expression of key intermediates in the *de novo* lipogenesis pathway, and fatty acid uptake and oxidation of wildtype (WT) and *Pon1* knockout (KO) mice fed with normal chow diet (NCD), high-fat diet (HFat) or high-fructose diet (HFruct). Data are presented as mean ± SEM for 5-10 mice. * $p<0.05$.

Glut2 expression is sensitive to dietary regimen while G6Pase expression is downregulated in the absence of PON1

Besides lipid metabolism, the liver has also an important role for glucose metabolism. While in the fed state, the liver is responsible for glucose storage, in the fasting state, the liver produces glucose from several substrates (Rui, 2014). *Glut2* expression was increased in NCD KO mice compared with NCD WT mice, as well as in HFat KO compared with HFat WT (Figure 21A). The opposite occurred in HFruct mice, where *Pon1* deletion led to a decrease of *Glut2*. Although there were no differences in

glucokinase expression, L-PK was increased in HFat mice, independent of the genotype, compared with both NCD and HFruct animals (Figure 21A-B). On the other hand, HFat KO mice presented decreased *Pepck* expression compared with NCD KO mice. Regarding the last step of gluconeogenesis, the absence of PON1 resulted in decreased expression of G6Pase, in both NCD and HFruct diet fed mice (Figure 21A-B).

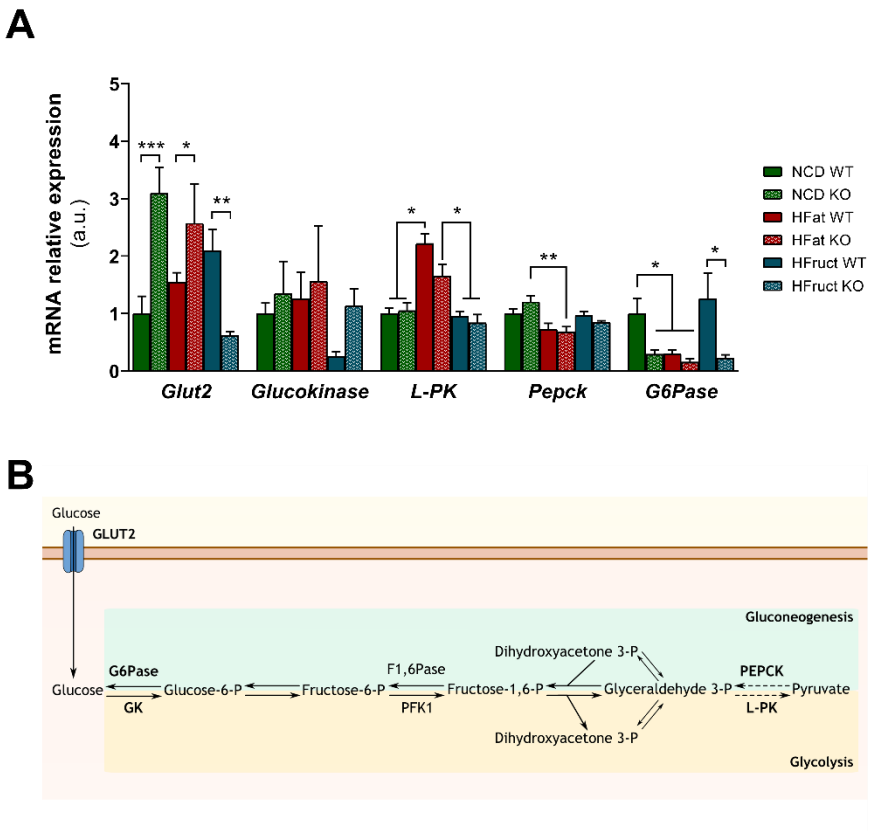

Figure 21 – Effects of *Pon1* deletion under Normal Chow Diet, High-Fat or High-Fructose feeding on the mRNA expression of glucose metabolism-related genes. Panel A - Gene expression of key intermediates in hepatic glucose metabolism of wildtype (WT) and Pon1 knockout (KO) mice fed with normal chow diet (NCD), high-fat diet (HFat) or high-fructose diet (HFruct). Panel A – Scheme of glycolysis and gluconeogenesis. G6Pase – glucose-6 phosphatase; GLUT2 – glucose transporter 2; GK – glucokinase; L-PK – pyruvate kinase; PEPCK - phosphoenolpyruvate carboxykinase; Data are presented as mean ± SEM for 5-7 mice. * $p<0.05$; ** $p<0.01$.

Discussion

The sedentary lifestyle and poor nutritional habits have led to an increased prevalence of metabolic disorders, as obesity, T2DM and NAFLD. Notably, these nutritional habits include the increased consumption of highly processed food resulting in increased intake of simple carbohydrates and saturated fat (Rippe and Angelopoulos, 2013). Although it is already known that the enrichment in lipids and carbohydrates, particularly fructose (Dekker *et al.*, 2010), has different physiological effects (Parry and Hodson, 2017), the role of certain endogenous antioxidant proteins, as PON1, in counteracting the deleterious effects of these components of the diet is not fully understood (Meneses *et al.*, 2019). As this enzyme is mainly produced by the liver and considering its antioxidant actions, we investigated the effects of PON1 deletion on overall glucose homeostasis and on hepatic metabolism, with a further insult of high-fat or high-fructose feeding.

The absence of PON1 had no effects in body weight gain, nor in the adipose tissue depots weight after 12 weeks of diet. Moreover, no differences were found in insulin sensitivity upon *Pon1* deletion, although, as expected, the different diets resulted in distinct degrees of insulin resistance. In contrast, glucose tolerance was affected by *Pon1* deletion in both NCD and HFat-fed mice. In fact, in these groups, *Pon1* deletion led to glucose intolerance, which points towards a role of PON1 on glucose homeostasis. As this occurred in both HFat and NCD groups, this metabolic dysregulation was independent of fat intake and obesity development. Other studies have found differences in both insulin resistance and glucose tolerance with normal chow diet and high-fat diet (Garcia-Heredia *et al.*, 2013, Koren-Gluzer *et al.*, 2013). However, in those studies the composition of the diets is not described and thus, it may differ in components (or their concentration) in the diet that are known to affect insulin sensitivity, as is the case of saturated fats (Buettner *et al.*, 2006).

Due to the detected differences in glucose homeostasis, insulin levels were also measured to assess insulin homeostasis. The measurement of C-peptide levels is the most reliable method to evaluate insulin secretion, even more than measuring insulin levels (Van Cauter *et al.*, 1992). Insulin has a lower half-life (of about 6 min vs 30 min) and suffers the first-pass effect in the liver, which takes more than 70% of the secreted insulin from the circulation (Duckworth *et al.*, 1998). C-peptide levels were increased in KO mice, under both NCD and HFat diets. However, insulin levels were not affected by the absence of PON1, which points towards increased hepatic insulin clearance as insulin levels are the result of the balance between its secretion and clearance (Najjar and Perdomo, 2019). Nonetheless, the hypothesis that these mice may have decreased C-peptide clearance, rather than increased insulin clearance cannot be excluded. Indeed, contrary to insulin, C-peptide clearance occurs in the kidney, thus renal impairment may cause impairments in C-peptide clearance, leading to increased C-peptide levels in the circulation (Jaspan *et al.*, 1977). In fact, patients with chronic kidney disease have lower levels of PON1 as well as lower PON1 activity in the serum (Miljkovic *et al.*, 2018), indicating that PON1 might play a key role in the maintenance of proper kidney function.

Insulin signaling and nutrient levels are crucial to coordinate the hepatic metabolic response to feeding. This signaling is mediated by InsR, where insulin binds, leading to the autophosphorylation of the receptor. Further transduction of this pathway occurs through Akt phosphorylation (Patel and

Goyal, 2019, Rui, 2014). Indeed, upon insulin stimulation, the levels of pInsR did not change in KO mice compared to the WT, in any of the diets. However, there was a marked increase in pAktT308 levels in NCD KO mice compared with NCD WT mice. While the same occurred in HFruct KO mice, but in both phosphorylation sites of Akt, in HFat mice, no differences were observed. As HFat diet per se causes insulin resistance and impairment of the insulin signaling pathway (Leclercq *et al.*, 2007), *Pon1* deletion in these mice did not exert any effect in the insulin signaling pathway. In NCD KO and HFruct KO, the increased phosphorylation of Akt, either in serine or threonine residues may indicate an overactivation of this pathway. However, we cannot disregard that Akt then acts on multiple downstream targets, as glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin or forkhead box O1 (Patel and Goyal, 2019). Therefore, Akt activation may not be only due to insulin signaling but also with some other stimuli. Indeed, it was found that interleukin-6 phosphorylates Akt at Ser473 residue and activates *Pon1* gene expression in HepG2 cells (Cheng *et al.*, 2013). This could explain the increase in Akt phosphorylation as an attempt to increase PON1 levels in the KO mice, but further studies will be needed to confirm this hypothesis *in vivo*.

Given the association between both PON1 levels and activity and lipoproteins, total cholesterol and triglycerides were assessed in both serum and liver tissue. No differences were observed in circulating FFAs, triglycerides nor cholesterol, which is in accordance with a previous study (Shih *et al.*, 1998). Regarding the liver, there were no differences in the hepatic content of triglycerides and cholesterol. Furthermore, the analysis of liver histological sections showed no differences in ectopic fat deposition upon *Pon1* deletion, in neither of the diets. These results are in accordance with a previous study that found no differences in PON1 KO mice fed with normal chow diet (Garcia-Heredia *et al.*, 2013). Although *Pon1* deletion has already been associated with severe liver steatosis, this might have been due to the intake of a high-fat high-cholesterol diet, a diet used to promote ectopic fat accumulation in the liver, rather than by PON1 absence per se (Garcia-Heredia *et al.*, 2013). Although there are no differences in free fatty acids levels, the expression of CD36, or fatty acid translocase, was increased in HFruct mice, independent of *Pon1* deletion. Moreover, *Pon1* deletion caused a downregulation of *Scd1* in mice fed with HFat diet, which may point towards a role of PON1 in lipogenesis. However, as liver metabolism was assessed after an overnight fast, it is anticipated that the lipid-related metabolism is downregulated, and any significant differences may have been blunted by the fasting state (Rui, 2014). In this case, the expected predominant metabolic activity encompasses gluconeogenesis to generate glucose for release to the circulation and the use of fatty acids coming from adipose tissue lipolysis for ketogenesis (Rui, 2014). Indeed, no differences were found in *Srebp2* expression levels, a transcription factor that regulates lipid metabolism. On the contrary, the expression of *Chrebp*, that regulates gene transcription related to both glucose and lipid metabolism, was increased in NCD mice upon *Pon1* deletion. On the other hand, the absence of PON1 upon HFruct decreased *Chrebp* expression. Although the expression of *Chrebp* is increased, it may not be activated. Indeed, *Chrebp* is inhibited by several metabolites produced during fasting, as is the case of ß-hydroxybutyrate and acetoacetate, promoting its cytosolic localization (Nakagawa *et al.*, 2013).

Chrebp is known to upregulate the expression of L-PK, the last enzyme in glycolysis that culminates in the formation of pyruvate (Ortega-Prieto and Postic, 2019). However, in this study, this was not confirmed, as L-PK was only increased in HFat mice, and independently of PON1. In fact, as L-PK is stimulated by insulin, and HFat mice have increased insulin levels even in the fast state, these results are in accordance with the previous ones. Moreover, G6Pase was decreased in the three diets upon *Pon1* deletion, which indicates that PON1 might have a role in hepatic glucose production, as G6Pase hydrolyzes glucose-6-phosphate into glucose, allowing the latter to be exported to circulation by glucose transporters. Interestingly, *Glut2* expression was increased in KO mice, but only under NCD. In fact, under HFruct diet, *Glut2* was decreased upon PON1 absence, having the opposite result comparing to NCD. Although GLUT2 is essential for hepatic glucose uptake, it is not for glucose export. Studies suggest that glucose may be released through a membrane traffic-based pathway as GLUT2 deletion did not impair hepatic glucose production (Guillam *et al.*, 1998).

In conclusion, this study provides evidence that the absence of PON1 impacts on glucose homeostasis, without a clear effect in the development of obesity or NAFLD. Moreover, it highlights the different metabolic changes that high-fat or high-fructose feeding promote, and the role that PON1 may have upon these challenges. In fact, to our knowledge, this is the first study using a high-fructose diet in PON1 KO animals. In HFruct mice, although the absence of PON1 did not cause alterations in glucose tolerance, as in NCD and HFat mice, it caused alterations in hepatic glucose metabolism, namely in the expression of *Glut2* and *G6Pase*. These findings extend the understanding about PON1, also raising new questions about the effect of this important antioxidant protein on hepatic and renal metabolism.

Paraoxonase-1 and Apolipoprotein J as biomarkers in dysmetabolism: a cluster analysis in a subpopulation of PREVADIAB2

Abstract

Metabolic disorders are characterized by a prooxidant and proinflammatory environment. Decreased levels of paraoxonase 1 (PON1), an enzyme with anti-inflammatory and antioxidant actions, have been associated with type 2 diabetes *mellitus* (T2DM), high low-density lipoprotein-cholesterol (LDL-c), and renal insufficiency. PON1 is mainly produced in the liver and secreted to the bloodstream as a high-density lipoprotein (HDL) constituent, where apolipoprotein J (ApoJ) also coexists, having a protective role on atherosclerosis. ApoJ has already been associated with insulin resistance. Stratification of dysmetabolic profiles, even using common features, can allow a refined dysmetabolism diagnosis and prognosis leading to better clinical outcomes. We hypothesized that PON1 activity (POase) and ApoJ levels can be used as co-biomarkers in early stages of dysmetabolism.

A cluster analysis was performed in individuals from the PREVADIAB2 cohort. 304 individuals were analyzed: 139 with normoglycemia, 126 with prediabetes (86 IGT; 25 IFG; and 15 IFG+IGT) and 39 T2DM. We used a hierarchical algorithm informed by POase activity, ApoJ levels, age, estimated glomerular filtration rate (eGFR; CKD-EPI), high-density lipoprotein cholesterol (HDL-c), LDL-c, and triglycerides. The optimal number of clusters was determined using silhouette index.

We identified four groups, three grouping individuals with dysmetabolism, and one mainly with healthy younger people. However, the three clusters with most individuals with dysmetabolism could be further distinguished. One of them presents low POase activity and includes mainly subjects with dysglycemia, dyslipidemia, insulin resistance, and higher eGFR, suggesting hyperfiltration to compensate for dysmetabolism. The other two clusters were differentiated by POase activity. However, they were similar in other evaluated parameters, having in general intermediate levels in the other parameters when compared to the previously mentioned clusters. This suggests distinct genetic backgrounds or pathophysiological mechanisms.

The (dys)metabolic profiles identified can be linked to what is observed in clinical practice. Hence, using POase activity a co-biomarker of currently used parameters might allow a refinement of dysmetabolism diagnosis.

Keywords: Diabetes, Obesity, Dyslipidemia, Cluster Analysis, Paraoxonase-1, Dysmetabolism

Introduction

Diabetes, besides common features such as impaired insulin deficiency and/or action with a consequent increase in blood glucose levels, is a highly heterogeneous disease. Even in the prodromal stage of the disease, prediabetes, individuals may already present a wide variety of other metabolic disorders, such as dyslipidemia, non-alcoholic fatty liver disease (NAFLD), or nephropathy (Buysschaert *et al.*, 2015). Moreover, the kidney has an important role in homeostasis and in the last years there has been a growing interest in the kidney's role for dysmetabolism and on the liver-kidney axis. Indeed, an impairment of this axis may lead to the development of the nephrotic syndrome and hepatorenal syndrome due to dyslipidemia and hepatic dysfunction, respectively (Raj *et al.*, 2020).

Although they share specific features, one of the common hallmarks of metabolic disorders is the pro-inflammatory and prooxidant *milieu*. Paraoxonase-1 (PON1) is an endogenous antioxidant and anti-inflammatory protein (Furlong *et al.*, 2016). After being produced in the liver, PON1 is released to the bloodstream, where it is found, not only, but mostly, on HDL (Fuhrman *et al.*, 2005, Gaidukov and Tawfik, 2005). In fact, PON1 is partly responsible for the HDL antioxidant properties, participating in the inhibition of LDL oxidation. Moreover, PON1 prevents atherosclerosis by inhibiting monocyte chemoattractant protein 1 (MCP1) production, a consequence of oxidized LDL action in endothelial cells (Mackness *et al.*, 2004). Some studies have observed that PON1 activity might be decreased in patients with diabetes, as hyperglycemia impacts on PON1 binding capacity to HDL (Mackness *et al.*, 2000). These particles are also known to have apolipoproteins, as is the case of apolipoprotein J (ApoJ). The latter is a protein with several distinct roles, namely chaperone activity towards PON1 (De Silva *et al.*, 1990). Indeed, ApoJ participates in the maintenance of proper protein conformation by binding to misfolded proteins, therefore promoting their clearance from the extracellular space (Rull *et al.*, 2015). Like PON1, it has already been hypothesized that it may have a protective role in atherosclerosis, for its antioxidant role towards LDL (Rull *et al.*, 2015). As complex diseases lead to complex phenotypes, individuals with the same condition do not present the same symptoms, neither the same clinical course and not even the same treatment response (McCarthy, 2017, Merino and Florez, 2018). Thus, it is imperative to better comprehend and evaluate disease heterogeneity and clinical outcomes. The development of new tools and the discovery of new biomarkers, that allow to stratify and distinguish different phenotypes in terms of etiology, severity or underlying mechanisms represents a challenge but is crucial for better disease prognosis.

Herein, we hypothesized that serum PON1 activity and ApoJ levels may be used as co-biomarkers in early stages of dysmetabolism. The aim of this work was to stratify a subpopulation of the PREVADIAB2, the Portuguese diabetes prevalence study (Gardete-Correia *et al.*, 2010), relative to PON1 activity, ApoJ levels, age, lipid profile and renal function.

Material and Methods

Population

All subjects voluntarily provided written informed consent for participation in the PREVADIAB2. The study protocol and all associated procedures were approved by the Ethics Committee of the APDP – Diabetes Portugal and NOVA Medical School (CEFCM/02/2020), and by the Portuguese Data Protection Authority (3228/2013). From the PREVADIAB2 population, 304 individuals were included in this study: 139 with normoglycemia, 126 with prediabetes (86 IGT; 25 IFG; and 15 IFG+IGT) and 39 with T2DM. The glycemic thresholds were defined using WHO/International Diabetes Federation (IDF) glycemia criteria.

Clinical measurements

Participants were weighed to the nearest 0.01 Kg and their waist circumference was measured at the midline between the inferior limit of the ribs and the top of the iliac crest. Height was measured to the nearest 0.1 cm with a stadiometer according to the WHO procedures. Blood collection was performed after an overnight fast, immediately before and during an oral glucose tolerance test (OGTT; 0, 30 and 120 min). The samples were then centrifuged at 3500 rpm at 4°C for 10 min and plasma and serum samples were kept at -80°C until further analysis. Serum samples were used to analyze the lipid profile of the individuals, including total, LDL and HDL cholesterol, free fatty acids (FFA), and triglycerides (TG) and to quantify hepatic markers as gamma-glutamyltransferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), using enzymatic tests in an automated chemistry analyzer (Auto Analyzer Olympus AU640, Beckman Coulter). Non-HDL cholesterol was calculated as total cholesterol minus HDL. Glycated hemoglobin (HbA1c) was determined by HPLC with boronate affinity (Menarini Premier Hb 9210). Plasma glucose levels were measured with a glucose analyzer (glucose oxidase method, Olympus AU640, Beckman Coulter). Plasma insulin and C-peptide levels were determined by chemiluminescence assays (DiaSorin, Italy).

Serum Paraoxonase activity

Serum paraoxonase (POase) activity was measured as previously described (Batuca *et al.*, 2007). Paraoxon (1.0 mM; Sigma-Aldrich) was freshly prepared in 290 µL of 50 mM glycine buffer containing 1 mM calcium chloride (pH 10.5). Then, the latter was incubated with 10 µL of serum at 37°C for 40 min in 96-well plates. p-Nitrophenol formation was monitored at 412 nm and activity expressed in U per L of serum.

Serum ApoJ Levels

ApoJ levels were measured through Human Clusterin Quantikine ELISA Kit (R&D Systems, MN, USA) following manufacturers' instructions. Briefly, serum samples were diluted and incubated for 2 h at room temperature in a plate shaker along with the standard curve and controls. Then, the plate was washed, and Human Clusterin Conjugate was added. The plate was incubated at room temperature

for another 2 h in a plate shaker. The plate was washed, and the substrate solution was added and incubated for 30 min without shaking. Stop solution was then added and the optical density of each well was measured at 540 nm and 450 nm. Results were obtained after averaging the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Then, the four-parameter logistic standard curve was generated, and the samples' optical densities were plotted.

Metabolic and functional indexes

BMI was calculated as weight (Kg) / [height (m)]^2. The three time points of the OGTT (0, 30 and 120 min) were used to determine the area under the curve (AUC) of glucose, FFA, insulin and C-peptide excursions using the trapezoidal rule (Le Floch *et al.*, 1990). NAFLD-Fatty Liver Score (NAFLD-FLS) was used as a surrogate index to evaluate fatty liver and was calculated as −2.889 + 1.179 x IDF metabolic syndrome (yes=1/no=0) + 0.454 x type 2 diabetes (yes=2/no=0) + 0.145 x $Insulin_{0\ min}$(mIU/L) (Balkau *et al.*, 2010). Tissue-specific insulin resistance indexes were used to profile the clusters. Hepatic-IR was calculated as $Insulin_{AUC(0\text{-}30)}$ x $Glucose_{AUC(0\text{-}30)}$ (Abdul-Ghani *et al.*, 2007). Adipo-IR was calculated as FFA_{0min} x $Insulin_{0min}$ (Gastaldelli *et al.*, 2009). Finally, we estimated the glomerular filtration rate using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula, where GFR = 141 × min(serum creatinine/K, 1)$^{\alpha}$ × max(serum creatinine/K, 1) −1.209 × 0.993^{Age} × 1.018 [if female] and/or 1.159 [if black]; K is 0.7 for females and 0.9 for males, α is −0.329 for females and −0.411 for males, and min indicates the minimum of serum creatinine /K or 1, and max indicates the maximum of serum creatinine/K or 1 (Levey *et al.*, 2009).

Cluster Analysis

Serum POase activity and ApoJ levels, age, estimated glomerular filtration rate (eGFR; CKD-EPI), HDL, LDL and TAG levels were used to inform the clustering algorithm and therefore stratify the population. These parameters were chosen as they may be easily measured in clinical practice. Moreover, we hypothesize that both POase activity and ApoJ levels may refine the clustering and unveil more phenotypes that using only the other parameters. First, the parameters were scaled. Then, the cluster analysis was made using a hierarchical algorithm (Euclidean distance, *ward method*) hclust in R and the optimal number of clusters was determined based on the silhouette width index.

Statistical Analysis

Experimental data are shown as Tukey box plots unless stated otherwise. Statistical analysis was performed using two-way ANOVA in Graphpad Prism 8 (GraphPad Software, San Diego, CA, USA) with Bonferroni correction. Correlations were analyzed using Spearman correlation. $p<0.05$ was considered significant.

Results

In the present study, 304 subjects of the PREVADIAB2 cohort were analyzed. This population had a mean age of 62 years and 61% of them were women. Of these individuals, and according to WHO/IDF guidelines (International Diabetes Federation, 2017), 45% had normoglycemia, 41% prediabetes (28% IGT; 8% IFG; 5% IFG+IGT) and 13% had T2DM (Table 6). Herein, and when grouping the individuals by their glycemic phenotype, we observed no differences regarding age between the different groups. As expected, there was a significant difference in fasting glycemia between NGT and both IFG and IFG+IGT ($p<0.001$). Moreover, there were significant differences in both fasting glycemia and glycemia 2 h after the OGTT between NGT and T2DM ($p<0.001$). In fact, 2 h-post OGTT glycemia was significantly different in NGT individuals compared to all the other phenotypes ($p<0.001$), except for IFG (Table 6). Total cholesterol, LDL-c, and triglycerides were significantly different between NGT and T2DM ($p<0.05$, $p<0.05$, and $p<0.001$, respectively). Moreover, we observed that POase activity was decreased in IGT individuals when compared to NGT (Table 6; Figure 22). However, ApoJ levels did not present any significant differences among the different groups (Table 6; Figure 22).

Table 6 – Population characterization by glycemic phenotype. Summary of the population results grouped by glycemic phenotype. Lines in grey highlight the parameters that were used to inform the clustering algorithm.

		All	NGT	IGT	IFG	IFG+IGT	T2DM
N (%)		304	139 (45)	86 (28)	25 (8)	15 (5)	39 (13)
Female, n (%)		186 (61)	82 (59)	60 (70)	9 (36)	10 (67)	25 (64)
Age (y)	Mean	62.21	58.48	65.12[a]	62.56	66.60	67.18[a]
	SEM	0.75	1.29	1.11	1.21	2.19	1.79
BMI (Kg/m^2)	Mean	27.82	27.02	27.51	29.29	28.93	29.96[a]
	SEM	0.27	0.39	0.48	0.79	1.04	0.82
Glycemia Fasting (mg/dL)	Mean	99.29	92.39	93.76	115.42[a,b]	115.01[a,b]	119.71[a,b]
	SEM	0.95	0.83	1.00	0.91	0.96	4.15
Glycemia 2 h post-OGTT (mg/dL)	Mean	138.91	103.30	160.02[a]	110.82[b]	166.81[a,c]	226.52[a,b,c,d]
	SEM	2.88	1.83	1.68	3.94	2.88	9.14
HbA1c (%)	Mean	5.73	5.53	5.65	5.93[a]	6.07[a,b]	6.31[a,b,c]
	SEM	0.03	0.03	0.04	0.07	0.13	0.13
Cholesterol (mg/dL)	Mean	201.29	200.17	197.94	200.28	199.95	213.79
	SEM	2.15	2.95	3.97	8.98	6.89	7.16
HDL (mg/dL)	Mean	52.75	53.77	52.16	51.16	52.03	51.68
	SEM	0.68	0.95	1.07	2.76	3.31	2.48
LDL (mg/dL)	Mean	137.23	136.03	134.41	137.56	137.00	147.62
	SEM	1.75	2.40	3.41	6.98	6.90	5.37
TAG (mg/dL)	Mean	125.77	107.51	130.36	160.09[a]	128.98	157.50[a]
	SEM	4.06	4.53	7.65	22.26	13.72	12.92
eGFR (mL/min/1.73 m^2)	Mean	72.27	73.26	73.39	71.13	71.10	67.41
	SEM	0.80	1.22	1.34	2.61	3.95	2.53
POase Activity (U/L)	Mean	141.42	151.12	128.72[a]	138.45	135.98	140.07
	SEM	2.62	3.97	4.85	7.37	12.21	6.69
ApoJ Levels (µg/mL)	Mean	308.70	320.02	319.85	280.52	293.85	264.51
	SEM	10.82	15.54	25.25	23.68	28.85	23.41

Significantly different results ($p<0.05$) are indicated as: a vs. NGT; b vs. IGT; c vs. IFG, d vs. IFG+IGT. NGT – normal glucose tolerance; IGT – impaired glucose tolerance; IFG – impaired fasting glucose; T2DM – type 2 diabetes *mellitus*.

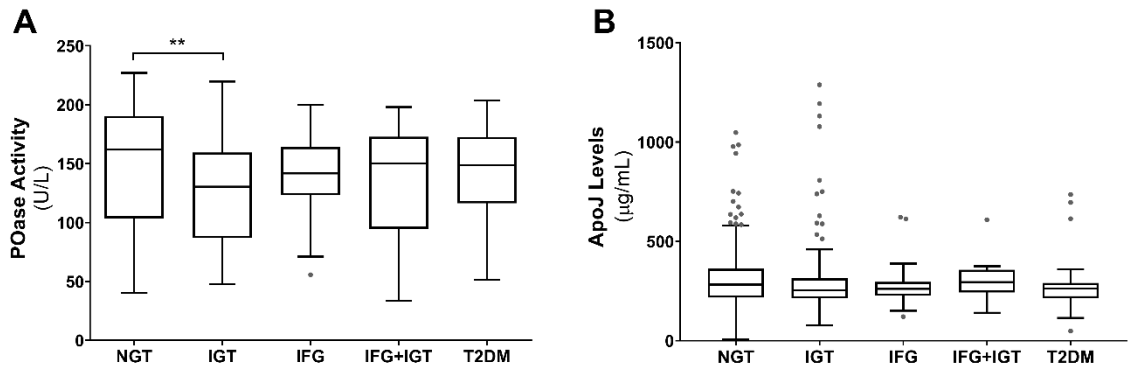

Figure 22 – Serum paraoxonase activity and apolipoprotein J levels across glycemic phenotypes. Serum paraoxonase (POase) activity (Panel A) and Apolipoprotein J (ApoJ) levels (Panel B) in individuals with normoglycemia (NGT), impaired glucose tolerance (IGT), impaired fasting glucose (IFG), impaired fasting glucose and impaired glucose tolerance (IFG+IGT) or type 2 diabetes *mellitus* (T2DM). Significantly different results are indicated as: ** $p<0.01$.

After informing the analysis with serum PON1 activity and ApoJ levels, age, estimated glomerular filtration rate (eGFR; CKD-EPI), HDL-c, LDL-c and TAG, the optimal number of clusters was 4 (Cluster A: 26, Cluster B: 19, Cluster C: 105, and Cluster D: 154 individuals). All the clusters encompass individuals with dysglycemia, although cluster A has mostly individuals with normoglycemia (96%), having only 1 IGT individual (Figure 23A). Additionally, cluster B is the cluster with more dysglycemic individuals and cluster C encompasses more IGT individuals than the overall population (Figure 23A). Concurrent with glycemic phenotype, cluster D also has a gender distribution comparable to the overall population, as well as cluster C. On the contrary, cluster B has more males and cluster A more females than the overall (Figure 23B). Regarding age, clusters A and B have significantly younger individuals compared with clusters A and B, being the individuals of cluster A even younger that individuals in cluster B (Figure 23C). Regarding BMI, there are no significantly differences between the clusters (Figure 23D).

Regarding POase activity, cluster A and D present higher POase activity, while clusters B and C present lower POase activity (Figure 24A). For ApoJ levels, cluster D has significantly higher levels of ApoJ than clusters A and C (Figure 24B). Regarding lipid profile, cluster A, the cluster with more normoglycemic subjects, has lower levels of non-HDL-c and higher levels of HDL-c compared to the cluster with more dysglycemic individuals (Cluster B). In concurrence with the higher prevalence of dysglycemia in cluster B, this is also the cluster with higher levels of total cholesterol, non-HDL-c and triglycerides, being also the one with lower levels of HDL-c (Figure 24D-F).

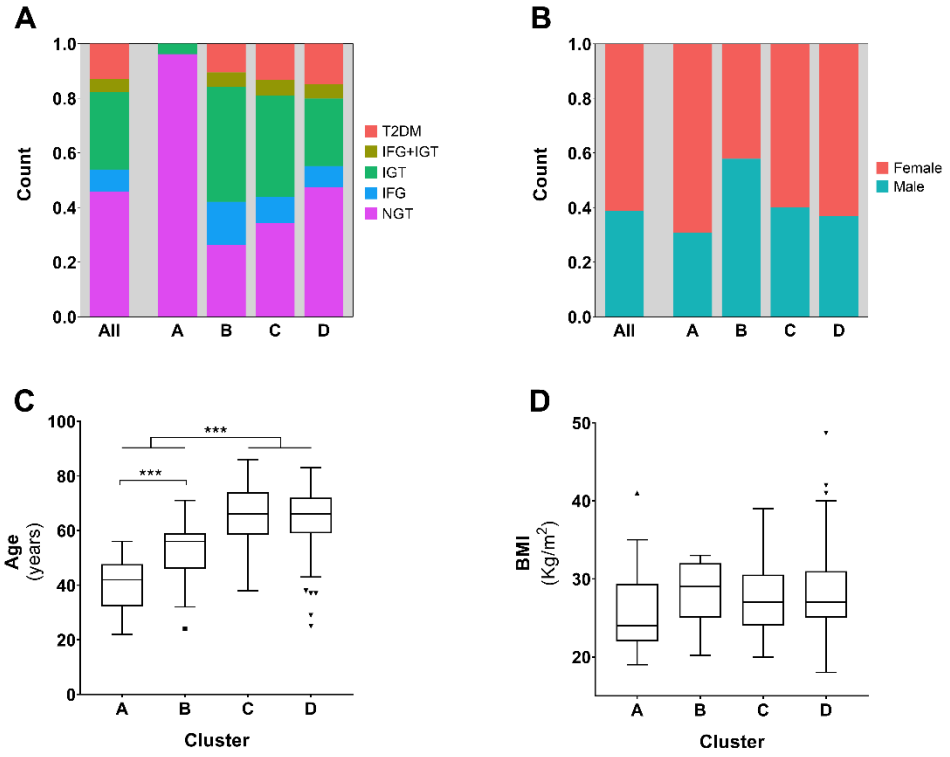

Figure 23 - Clusters characterization. Distribution of glycemic phenotypes (Panel A), gender (Panel B), age (Panel C) and body mass index (BMI; Panel D) by cluster. Significantly different results are indicated as: *** p<0.001.

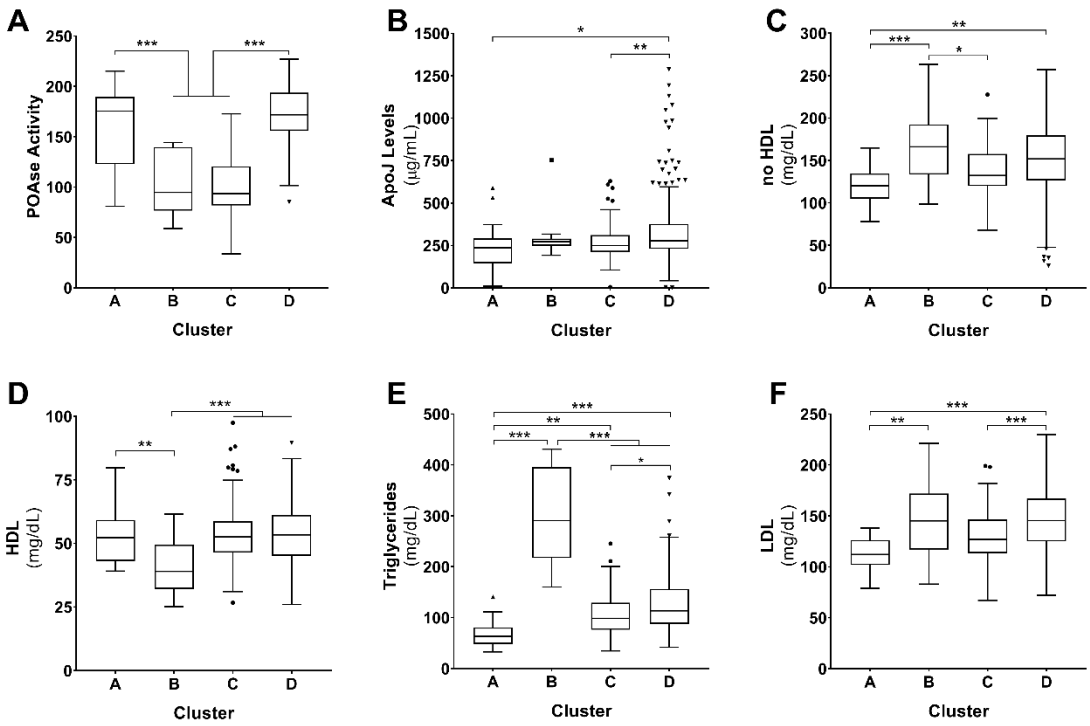

Figure 24 - Cluster paraoxonase-1 activity, apolipoprotein J levels and lipid profile. Cluster paraoxonase-1 activity (POase; Panel A); apolipoprotein J (ApoJ) levels (Panel B); non-HDL levels (Panel C); HDL, triglycerides and LDL levels (Panel D, E and F, respectively). Significantly different results are indicated as: * p<0.05; ** p<0.01; *** p<0.001.

During the OGTT, C-peptide levels, the fittest measurement for endogenous insulin secretion (Van Cauter *et al.*, 1992), were significantly higher in cluster B compared to all the other clusters. By the end of the OGTT, after 120 min of the glucose load, clusters C and D presented intermediate levels of C-peptide when compared to both cluster B, that had higher levels, and cluster A, that presented lower levels of C-peptide (Figure 25A). Insulin levels, and similar to C-peptide, were higher in cluster B compared to the other clusters throughout the OGTT (Figure 25B). Cluster A, having mostly NGT individuals, was the cluster with lower glucose levels during the OGTT (Figure 25C). Cluster B presented a similar profile to clusters C and D, demonstrating that individuals in cluster B needed to secrete more insulin to achieve the same glycemia, pointing towards the presence of insulin resistance. During fasting, and as a result of energetic demand, peripheral lipolysis is expected to occur leading to increased FFA in circulation. In the fed state, peripheral lipolysis should be suppressed, namely by increasing insulin levels (Burns *et al.*, 1963). Indeed, FFA levels were suppressed during the OGTT in every cluster. However, this decrease was more evident in cluster A, and less pronounced in cluster B (Figure 25D). These results point towards an efficient lipolysis regulation in cluster A and an impaired regulation by cluster B.

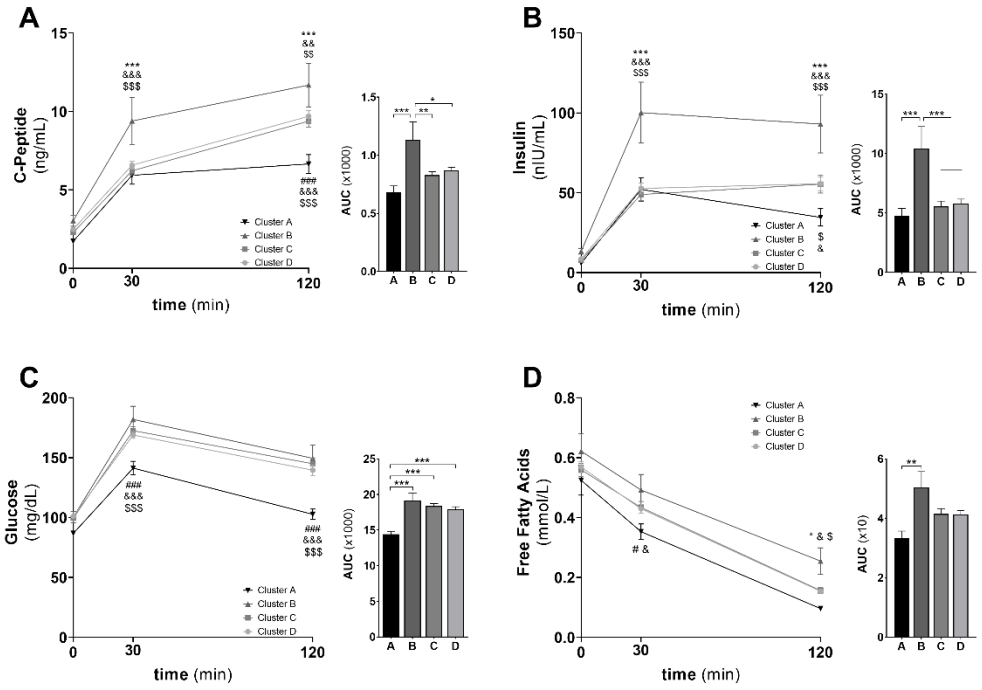

Figure 25 – Metabolic excursions during the oral glucose tolerance test. C-Peptide (Panel A); Insulin (Panel B); Glycemia (Panel C) and Free Fatty Acids (Panel D) levels during the oral glucose tolerance test (OGTT). * p<0.05 vs. Cluster A; ** p<0.01 vs. Cluster A; *** p<0.001 vs. Cluster A; # vs. Cluster B; & vs. Cluster C; $ vs. Cluster D.

Considering the differences in insulin and glucose excursions during the OGTT, surrogate indexes of insulin resistance and fatty liver were evaluated. Cluster A has significantly lower NAFLD-FLS, which is in accordance with the lower number of dysglycemic individuals and with the lower triglycerides and non-HDL cholesterol levels (Figure 26A). Moreover, and in accordance with the previous results

pointing towards dysmetabolism, cluster B is the cluster with higher Hepatic-IR and Adipo-IR, surrogate indexes that correlate with insulin resistance in the liver and adipose tissue, respectively (Figure 26B-C).

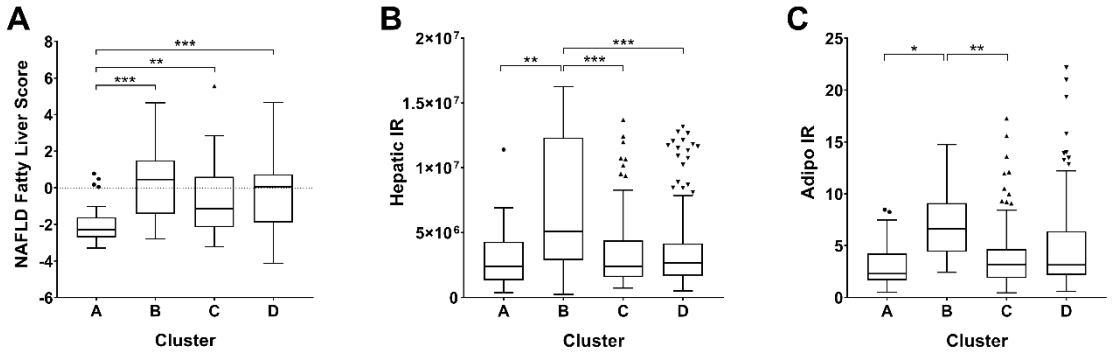

Figure 26 – Cluster NAFLD fatty liver score and hepatic- and adipo-insulin resistance indexes. Cluster Non-Alcoholic Fatty Liver Disease (NAFLD)-Fatty Liver Score (Panel A); Hepatic Insulin Resistance (IR) Index (Panel B); and Adipo IR Index (Panel C). Significantly different results are indicated as: * p<0.05; ** p<0.01; *** p<0.001.

Due to the importance of kidney function for homeostasis, eGFR was assessed. Cluster A and B have increased eGFR when compared to clusters C and D (Figure 27A). As eGFR is known to be affected by age, and due to age differences between the 4 clusters, the normalization by the number of expected functional glomeruli at a specific age was performed. In this case, the increased eGFR in cluster B is maintained (Figure 27B), suggesting hyperfiltration to compensate the dysmetabolism depicted in the previous figures.

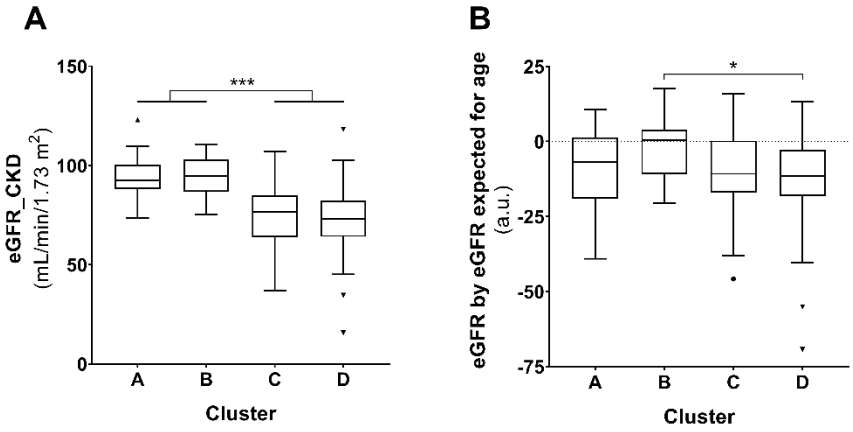

Figure 27 – Cluster estimated glomerular filtration rate. Panel A – Estimated glomerular filtration rate (CKD-EPI); Panel B – Estimated glomerular filtration rate normalized by expected for age. Significantly different results are indicated as: * p<0.05; *** p<0.001.

After undertaking the cluster analysis, and to further test our hypothesis, we performed Spearman correlations between all the variables analyzed during the PREVADIAB2 study in the overall population and within the four clusters. Specifically, we analyzed if PON1 activity and/or ApoJ levels correlated with the other parameters (Table 7). In the overall population, PON1 activity positively correlates

with ApoJ levels. Moreover, ApoJ levels positively correlate with HDL-c. When analyzing each cluster, these overall correlations disappear, due to the grouping of individuals by common characteristics. In cluster A, in which the individuals are younger than the overall population with no dyslipidemia and almost no dysglycemia, a positive correlation was found between PON1 activity and triglycerides, as well as a negative correlation between PON1 activity and HDL-c levels. In cluster B, ApoJ levels negatively correlate with surrogate indexes such as Adipo-IR, Hepatic-IR and NAFLD-FLS. Thus, although this is the cluster with higher levels of insulin resistance, the individuals that have increased ApoJ levels, have less tissue-specific IR. In cluster C, where individuals are older, have decreased PON1 activity and lower levels of ApoJ, compared to cluster D, and show no signs of dyslipidemia, there is a positive correlation between ApoJ levels and several lipid profile-related parameters, as LDL, HDL and non-HDL cholesterol. Moreover, PON1 activity negatively correlates with triglycerides. In cluster D, the cluster with older individuals, higher levels of PON1 activity and ApoJ levels and without dyslipidemia, there is a negative correlation between triglycerides and non-HDL cholesterol with PON1 activity and a negative correlation between ApoJ levels and LDL.

Table 7 – Correlations in the overall population and within the clusters. Spearman correlations between paraoxonase (POase) activity and/or Apolipoprotein J (ApoJ) levels and parameters related to tissue-specific insulin resistance indexes, kidney function and lipid profile in the overall population and within each specific cluster. NAFLD-FLS – Non-alcoholic fatty liver disease – fatty liver score; IR – insulin resistance; eGFR – estimated glomerular filtration rate; HDL – high-density lipoprotein; LDL – low-density lipoproteins.

	All		Cluster A		Cluster B		Cluster C		Cluster D	
	POase Activity	ApoJ Levels	POase Activity	ApoJ Levels	POase Activity	ApoJ Levels	POase Activity	ApoJ Levels	POase Activity	ApoJ Levels
NAFLD-FLS	–	–	–	–	–	r=-0.51; p=0.03	–	–	–	–
Hepatic-IR	–	–	–	r=0.50; p=0.009	–	r=-0.55; p=0.02	–	–	–	–
Adipo-IR	–	–	–	–	–	r=-0.49; p=0.03	–	–	–	–
eGFR CKD-EPI	r=-0.11; p=0.04	–	–	–	–	–	–	–	–	–
No-HDL	–	–	–	–	–	–	–	r=0.21; p=0.04	r=-0.19; p=0.02	–
HDL	–	r=0.15; p=0.008	r=-0.41; p=0.04	–	–	–	–	r=0.27; p=0.006	–	–
LDL	–	–	–	–	–	–	–	r=0.21; p=0.03	–	r=-0.18; p=0.03
Triglycerides	–	–	r=0.41; p=0.04	–	r=0.50; p=0.03	–	r=-0.25; p=0.01	–	r=-0.16; p=0.04	–
HOMA-IR	–	–	–	r=0.50; p=0.0096	–	–	–	–	–	–
ApoJ Levels	r=0.17; p=0.06	–	–	–	–	–	–	–	–	–

Discussion

Diabetes is a highly heterogeneous disease with numerous possible clinical courses (McCarthy, 2017, World Health Organization, 2017). Prediabetes and T2DM are known to be associated with increased risk to develop other metabolic disorders such as obesity, NAFLD, and cardiovascular disease, all characterized by inflammation and oxidative stress (Buysschaert *et al.*, 2015, Raposo, 2020, World Health Organization, 2017). However, and due to the heterogeneity of dysmetabolic profiles, a better understanding of the disease and the discovery of new biomarkers that could give earlier clues for dysmetabolism and better predict disease progression are of great interest (McCarthy, 2017, Neeland *et al.*, 2018).

Our data illustrates that there is no striking difference regarding serum POase activity and ApoJ levels between individuals with normoglycemia and dysglycemia, except for POase activity when comparing NGT and IGT individuals. Previous studies have observed increased ApoJ levels in individuals with T2DM in comparison with NGT individuals (Seo *et al.*, 2018). This study, however, disregarded individuals with prediabetes. Moreover, the NGT group was overweight, and the T2DM group was obese, with significantly higher BMI (Seo *et al.*, 2018). As metabolic disorders are overly complex, the difference in BMI might indicate different associated pathologies and thus confounding factors might exist. In fact, another study has found a relation between ApoJ levels and obesity, with obese individuals having increased levels of ApoJ (Won *et al.*, 2014). In our first approach, individuals were grouped by glycemic phenotype and all the groups were within the overweight classification ($25 \leq BMI < 30$), thus we could not verify this difference in ApoJ levels. In the population evaluated in this study, ApoJ levels positively correlate with both POase activity and HDL-c. Indeed, this might be explained by the presence of both proteins in HDL, and the function of ApoJ as chaperone of proteins like PON1 (Blatter *et al.*, 1993, De Silva *et al.*, 1990, Getz *et al.*, 2010).

To better assess the potential of these antioxidant proteins as possible biomarkers for dysmetabolism, we proceeded to a cluster analysis. This strategy allows to group individuals by similarity between them regarding the parameters used to inform the analysis instead of by clinically defined thresholds. In this study, besides POase activity and ApoJ levels, the individuals were grouped considering their age, lipid profile, and renal function. In this work, four groups were identified. Interestingly, all clusters were heterogeneous regarding glycemic phenotypes, combining subjects with normoglycemia, prediabetes and T2DM. This reveals that glycemia and, specifically, the glycemic phenotype was not a decisive factor to group the individuals. In fact, it emphasizes that groups of individuals with the same glycemic phenotype might still be heterogeneous. Cluster A encompasses the apparently healthy and young individuals of this population. Indeed, the individuals are younger than the overall population and do not present dyslipidemia, and dysglycemia is almost absent. Here, a positive correlation was found between POase activity and triglycerides, as well as a negative correlation between PON1 activity and HDL levels. Of evidence, within cluster A there is a correlation between ApoJ levels and insulin sensitivity (Table 7). These correlations demonstrate that, within this metabolically healthier cluster, the higher the POase activity is, the lower the HDL levels are, indicating that the antioxidant system is working and counteracting the growing levels of triglycerides.

Cluster B is a group that encompasses mostly dysglycemic individuals, with dyslipidemia, insulin resistance, and fatty liver. Furthermore, these individuals present glucose intolerance and increased insulin levels. Despite including normoglycemic individuals, this cluster presents a pronounced dysmetabolic profile. In accordance, cluster B is characterized by individuals with low POase activity and therefore with low antioxidant capacity to counteract dysmetabolism. This decrease might be related to the severity of metabolic abnormalities that the individuals present. Indeed, PON1 activity is affected by free radicals and overall oxidative stress, a hallmark of metabolic disorders (Senti *et al.*, 2003). Cluster B, along with cluster A, have increased eGFR. Of note, cluster B is the only cluster that presents higher eGFR than what is expected for the age of the individuals, indicating that these individuals may have glomerular hyperfiltration to compensate for the overall dysmetabolism (Tonneijck *et al.*, 2017). In this cluster, ApoJ levels negatively correlate with surrogate indexes such as Adipo-IR, Hepatic-IR, and NAFLD-FLS. Hence, within this cluster, individuals with higher levels of ApoJ are also the ones presenting lower values of the surrogated indexes of insulin resistance and fatty liver. Consequently, within cluster B subjects with higher ApoJ levels seem to be more protected against tissue-specific insulin resistance and NAFLD.

Clusters C and D were differentiated by POase activity and ApoJ levels, with cluster C having lower ApoJ levels and POase activity, but with similar age, lipid profile, eGFR, and glycemic, insulin and c-peptide excursions during the OGTT. These clusters encompass the older individuals, with an apparently normal lipid profile, and glycemic distribution like the overall population analyzed. In Cluster D, there is a negative correlation between triglycerides and non-HDL cholesterol with PON1 activity and a negative correlation between ApoJ levels and LDL. Given the anti-inflammatory and antioxidant effects of the two proteins and its higher levels in this cluster, it seems like there is a more efficient antioxidant system protecting from dyslipidemia. Moreover, the improved POase activity and ApoJ levels in cluster D might protect the aged population towards the dysmetabolic profile (Seo *et al.*, 2020). The difference in POase activity between cluster C and cluster D suggests distinct genetic backgrounds or pathophysiological mechanisms involved in these clusters. Specifically, several hypotheses arise for what cluster C individuals may represent. On one hand, cluster C can encompass subjects with PON1 polymorphisms. There are two known polymorphisms in the coding region of *Pon1*, Q192R and M55L, that are present in approximately 30% of the individuals (Hatzihidiroglou *et al.*, 2016, McDaniel *et al.*, 2014). Q192R polymorphism affects PON1 activity, being the Q allele responsible for decreased PON1 activity. In contrast, M55L leads to changes in protein levels, being the M allele responsible for a lower mRNA transcription, resulting in less protein and, consequently, may also lead to an overall decrease in serum POase activity (Brophy *et al.*, 2001, Gupta *et al.*, 2011). On the other hand, cluster C may represent individuals with HDL dysfunction, as is the case of individuals with kidney disease (Rysz *et al.*, 2020). Indeed, in kidney disease, HDL is dysfunctional although its levels might be within clinically defined normal values. The dysfunction is related to a lower antioxidant action and anti-inflammatory capacity, that can be explained by a reduction of PON1 activity (Kaseda *et al.*, 2015).

We have identified clusters with different glycemic profile, POase activity, and lipid levels, suggesting distinct pathophysiological significance. The identified (dys)metabolic clusters can be related to what is observed in clinical practice. Hence, using POase activity and ApoJ levels as co-biomarkers of currently used parameters might allow a refinement of dysmetabolism diagnosis. The impact of the founded profiles on cardiovascular risk, as well as the role of PON1 polymorphisms should be addressed in future studies. Importantly, this work gives new insights concerning the relevance of these two proteins, raising relevant and novel hypotheses to be evaluated.

Knockout of insulin-degrading enzyme leads to mice testicular morphological changes and impaired sperm quality

Abstract

Insulin-degrading enzyme (IDE) is a zinc metalloprotease responsible for degrading and inactivating several bioactive peptides, including insulin. Individuals without this enzyme or with a loss-of-function mutation in the gene that codifies it, present hyperinsulinemia. In addition, impairment of IDE-mediated insulin clearance is associated with the development of metabolic diseases, namely prediabetes. Although insulin regulates male fertility, the role of IDE on male reproductive function remains unknown. We proposed to study the influence of IDE in the reproductive potential of males. As insulin mediates key events for the normal occurrence of spermatogenesis, we hypothesized that IDE functioning might be linked with sperm quality. We used C57BL/6N mice that were divided in three groups according to their genotype: wildtype (WT), heterozygous and knockout (KO) male mice for *Ide*. Spermatozoa were collected from the cauda of epididymis and sperm parameters were evaluated. Testicular tissue morphology was assessed through hematoxylin and eosin stain. Mitochondrial complex protein levels and lipid peroxidation were also evaluated in the testicular tissue. Our results show that KO mice present a 50% decrease in testes weight compared to WT mice as well as a decrease in seminiferous tubules diameter. Moreover, KO mice present impaired sperm quality, namely a decrease in both sperm viability and morphology. These results provide evidence that IDE plays an important role in determining the reproductive potential of males.

Keywords: Insulin-degrading enzyme; Male fertility; Spermatozoa; Spermatogenesis; Testes

Introduction

Insulin degrading enzyme (IDE), also known as insulysin, is a thiol zinc-metalloendopeptidase that may be found in the cytosol, peroxisomes, endosomes, mitochondria and cell membrane (Leissring *et al.*, 2004, Vekrellis *et al.*, 2000). IDE is found in most organisms and is ubiquitously expressed independently of the sensitivity of the cells to insulin (Pivovarova *et al.*, 2016). Furthermore, its levels and conformation may be controlled by many signals, including cellular stress, glucagon, and free fatty acids (Tundo *et al.*, 2013, Wei *et al.*, 2014) supporting a multifunctional role of the protein and showing that IDE is finely tuned depending on the state of the cell. In fact, IDE was found to have a heat shock protein-like behavior and to interact with the ubiquitin-proteasome system (Tundo *et al.*, 2013). Due to its configuration, that changes between active and inactive states, IDE cleaves small peptides of assorted sequence, namely amyloid ß-protein (Farris *et al.*, 2003, Leissring and Selkoe, 2006) thus being closely related to Alzheimer's disease. Moreover, this metallopeptidase cleaves insulin, being considered the major intervenient in insulin regulation. Actually, IDE has a crucial role in insulin metabolism and in inhibiting insulin translocation and accumulation in the cell nucleus (Authier *et al.*, 1996, Harada *et al.*, 1993). Thus, loss-of-function mutations of IDE cause hyperinsulinemia (Farris *et al.*, 2004), one of the main features of prediabetes and type 2 diabetes *mellitus*. Inhibitors of IDE were already hypothesized as possible treatment for those disorders (Costes and Butler, 2014) but as IDE degrades other substrates besides insulin, this is a very complex topic.

Insulin regulation is crucial for several essential cellular processes, namely glucose uptake. Impairment of cell metabolism by insulin has a major impact in fertility, particularly in the reproductive potential of males. Insulin dysregulation, as occurs in prediabetes and other metabolic diseases, affects the nutritional support of spermatogenesis by changing glucose transport, acetate and lactate production and the expression of some metabolism-related genes in testicular cells (Alves *et al.*, 2012). These effects have a negative impact in sperm production and quality and are reported to be mainly mediated by Sertoli cells, the somatic testicular cells responsible for give physical and nutritional support to developing germ cells (Griswold, 1995). Data from several studies show that both prediabetes and diabetes *mellitus* alter sperm quality. In fact, decreased sperm concentration, motility and normal morphology (Amaral *et al.*, 2006, Schoeller *et al.*, 2012, Shrilatha and Muralidhara, 2007) and increased nuclear DNA damage (Mangoli *et al.*, 2013, Rama Raju *et al.*, 2012) have been reported in diabetic individuals. Moreover, mitochondria was also reported to be affected namely by an increase in mitochondrial DNA damage (Agbaje *et al.*, 2007) as well as altered sperm mitochondrial function (La Vignera *et al.*, 2015). However, the exact mechanisms by which insulin dysfunction mediates these effects remain largely unknown. Thus, it is imperative to study the impact of primary hyperinsulinemia derived from insulin clearance on male reproductive function. Herein, we hypothesized that IDE may be a key player in insulin dysfunction-associated male infertility.

Material and Methods

Chemicals

Mammalian Protein Extraction Reagent and BCA Protein Assay Kit were purchased from Thermo Scientific (Whalthan, MA, USA). Dried milk was purchased from Regilait (Saint-Martin-Belle-Roche, France). ECF™ substrate was purchased from GE Healthcare (Weßling, Germany). NZYColour Protein Marker II was purchased from NZYTech (Lisbon, Portugal). Coverquick 2000 was purchased from VWR (Radnor, PA, USA). Diff quick was purchased from Baxter Dale Diagnostics AG (Dubinger, Switzerland). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless stated otherwise.

Animals

C57BL6/N, full body *Ide* heterozygous mice were purchased from the European Mouse Mutant Archive (EMMA) and provided by Wellcome Trust Sanger Institute (Cambridge, United Kingdom). Heterozygous breeding generated the wildtype (WT), heterozygous (Het) and knockout (KO) animals used in the experiments. Animals were maintained on a 12 h light/dark cycle with standard chow diet (Special Diets Services, United Kingdom) and water *ad libitum* at Instituto Gulbenkian de Ciência (Oeiras, Portugal). Mice were monitored weekly for body weight, blood glucose levels, as well as for distress signals. All procedures followed ARRIVE guidelines and the Europeans laws (Directive 2010/63/EU) that rule the use of animals in research.

Tissue preparation

Testes were weighed to determine gonadosomatic index (GSI = [testes weight/body weight] × 100). The right testis was placed in Davidson's modified fixative and then dehydrated in a graded ethanol series, embedded in paraffin, cut into 4 µm sections, mounted onto glass slides and stained with hematoxylin & eosin. The diameter of 25 round seminiferous tubules per animal was measured in Cell^B (Olympus, Japan).

Evaluation of Sperm Parameters

The cauda of the left epididymis of each mouse was minced to allow sperm dispersion in Hank's balanced salt solution and sperm parameters were evaluated according to WHO indications (World Health Organization, 1999). To assess sperm motility, a drop of 100 µL of the sperm suspension was placed in a prewarmed (37°C) slide. The percentage of motile sperm was assessed under a light microscope (×100 magnification) in 10 random fields, and the average was used as the final motility. Sperm concentration was evaluated with a Neubauer chamber. Sperm viability was evaluated by the assessment of sperm membrane integrity using an eosin/nigrosin staining. A total of 5 µL of the sperm suspension obtained as described above was mixed with 5 µL of 0.5% eosin/nigrosin stain and placed on a prewarmed glass microscope slide. The number of viable and nonviable spermatozoa was determined by counting a total of 100 spermatozoa per slide in continuous random fields under a light microscope under ×1000 magnification, to determine the percentage of viable sperm (Rato *et al.*,

2013). Viable sperm remained white, whereas unviable sperm stained pink, due to increased membrane permeability, which allowed the dye uptake. Sperm morphology was assessed through Papanicolaou staining as described (World Health Organization, 1999).

Protein extraction

Total protein was extracted from testicular tissue using Mammalian Protein Extraction Reagent (supplemented with 1% protease inhibitor cocktail and 100 mM sodium orthovanadate) following the manufacturer's instructions. Protein concentration was determined by Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions.

Evaluation of Lipid Peroxidation

Lipid peroxidation was evaluated through the analysis of 4-hydroxynonenal (4-HNE) protein adducts by Slot Blot technique using a Hybri-slot manifold system (Biometra, Göttingen, Germany). The proteins (5 µg) were transferred to previously activated polyvinylidene difluoride (PVDF) membranes. The latter were then blocked for 90 min with 5% (w/v) non-fat milk and incubated overnight (4°C) with goat anti-4-HNE (1:5000, AB5605, Merck Millipore, Temecula, USA). Afterwards, membranes were incubated with rabbit anti-goat (1:5000, A4187, Sigma–Aldrich, St. Louis, MO, USA) for 90 min and then reacted with ECF™ and read using a Bio-Rad GelDoc XR (Bio-Rad, Hemel Hempstead, UK). Densities from each band were quantified using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK).

Evaluation of Protein Carbonylation and Nitration

Carbonyl groups are produced on protein side chains when oxidized and thus, carbonyl content is generally used as an indicator for protein oxidation. Protein nitration is a marker of inflammation, cell damage, and nitric oxide production and can be evaluated by measuring the resulting products, namely nitro-tyrosine. These parameters were evaluated using slot-blot technique as explained above. The resulting membranes were incubated overnight (4ºC) with rabbit anti-DNP (1:5000, D9656, Sigma–Aldrich, St. Louis, MO, USA) or rabbit anti-nitro-tyrosine (1:5000, 9691, Cell Signaling Technology, Leiden, Netherlands).

Western Blot

Protein samples (75 µg) were mixed with sample buffer (1.5% Tris, 20% glycerol, 4.1% sodium dodecyl sulfate (SDS), 2% ß-mercaptoethanol, 0.02% bromophenol blue, pH 6.8), sonicated for 10 min at 4ºC and denatured for 15 min at 55ºC. Proteins were fractionated in 15% polyacrylamide gels and electrophoresis was carried out for 85 min. The proteins were transferred from gels to activated PVDF membranes in a Mini Trans-Blot® cell (Bio-Rad, Hemel Hempstead, UK) and then blocked for 3 h in a 5% non-fat milk solution at room temperature. The membranes were then incubated overnight at 4ºC with MitoProfile® Total OXPHOS Antibody Cocktail (1:1000, MitoSciences, Oregon, USA, MS604). Mouse anti-ß-actin (1:5000, Thermo Scientific, Rockford, IL, USA, MA5-15739) was used as protein loading

control. The immune-reactive proteins were detected with goat anti-mouse IgG-AP (1:5000, A3562). Membranes were reacted with ECF™ and read with the Bio-Rad GelDoc XR (Bio-Rad, Hemel Hempstead, UK). Quantity One Software (Bio-Rad, Hemel Hempstead, UK) was used to obtain band densities, which were divided by the respective ß-actin band density and then normalized with the wild type group value.

Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

The total RNA (tRNA) of the samples was extracted using the EZNA Total RNA Kit (Omega Biotek, Norcross, USA) and its concentrations were determined by Nanodrop 2000 (ThermoFisher, Waltham, USA). RNAt was then reversely transcribed to complementary DNA (cDNA) as previously described (Rato *et al.*, 2013).

qRT-PCR to analyze the mRNA expression levels of *Bax*, *Bcl2* and *Casp3* was performed in a BioRad CFX96 system (Biorad, California, USA) using specific primers (Table 8). Amplification conditions comprised an initial denaturation step of 5 min at 95ºC, followed by 40 runs of a 3 steps cycle: a denaturation step of 30 s at 95ºC; an annealing step of 30 s with a specific temperature for each set of primers (Table 8); and an extension step of 1 min at 72ºC. The quantification cycle (Cq) was determined through cycle threshold and fold variation of the expression levels was calculated following Pfaffl's mathematical model (Pfaffl, 2001), using β-2-microglobulin and glyceraldehyde 3-phosphate dehydrogenase as reference genes.

Table 8 – List of primers used to evaluate apoptotic markers expression upon *Ide* deletion. Primer sequences and cycling conditions for polymerase chain reaction of B-cell lymphoma 2-associated X protein (*Bax*), B-cell lymphoma 2 (*Bcl2*), caspase 3 (*Casp3*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and ß-2-microglobulin (*B2M*).

Gene	Primer sequence (5' - 3')	Annealing temperature (ºC)	Amplicon size (bp)
Bax	Fwd: CTCAAGGCCCTGTGCACTAA Rvs: CAGCCACCCTGGTCTTGGAT	60	110
Bcl2	Fwd: GTGAACCTGGGGGAGGATTGT Rvs: CATGCTGGGGCCATATAGTTC	60	195
Casp3	Fwd: GAGCTTGGAACGGTACGCTAA Rvs: GAGTCCACTGACTTGCTCCC	59	118
Gapdh	Fwd: AAGAGGGATGCTGCCCTTAC Rvs: TACGGCCAAATCCGTTCACA	61	123
B2M	Fwd: GCTTCAGTCGTCAGCATGGC Rvs: GGATTTCAATGTGAGGCGGGT	57	187

Statistical Analysis

The statistical significance among the experimental groups was assessed by one-way ANOVA. All experimental data are shown as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 (GraphPad software, San Diego, CA, USA). Possible outliers were removed using Grubbs' method, $\alpha=0.2$. $p<0.05$ was considered significant.

Results

Effect of the absence of insulin-degrading enzyme on testicular morphology

Testes weight was decreased in Het mice (91.75 ± 9.38 mg) and further decreased in the KO mice (59.75 ± 2.59 mg) when compared with the WT group (121.03 ± 1.47 mg; Figure 28A). Concomitantly, the GSI which is a measure that links the body weight to the gonads weight was decreased in Het (0.54 ± 0.04%) and *Ide* KO mice (0.44 ± 0.06%) when compared to WT (0.73 ± 0.03; Figure 28B). However, when observing the seminiferous tubules diameter, only *Ide* KO mice presented a significant decrease (185.56 ± 4.71 µm) when compared to the WT group (214.92 ± 7.11 µm; Figure 28C-D).

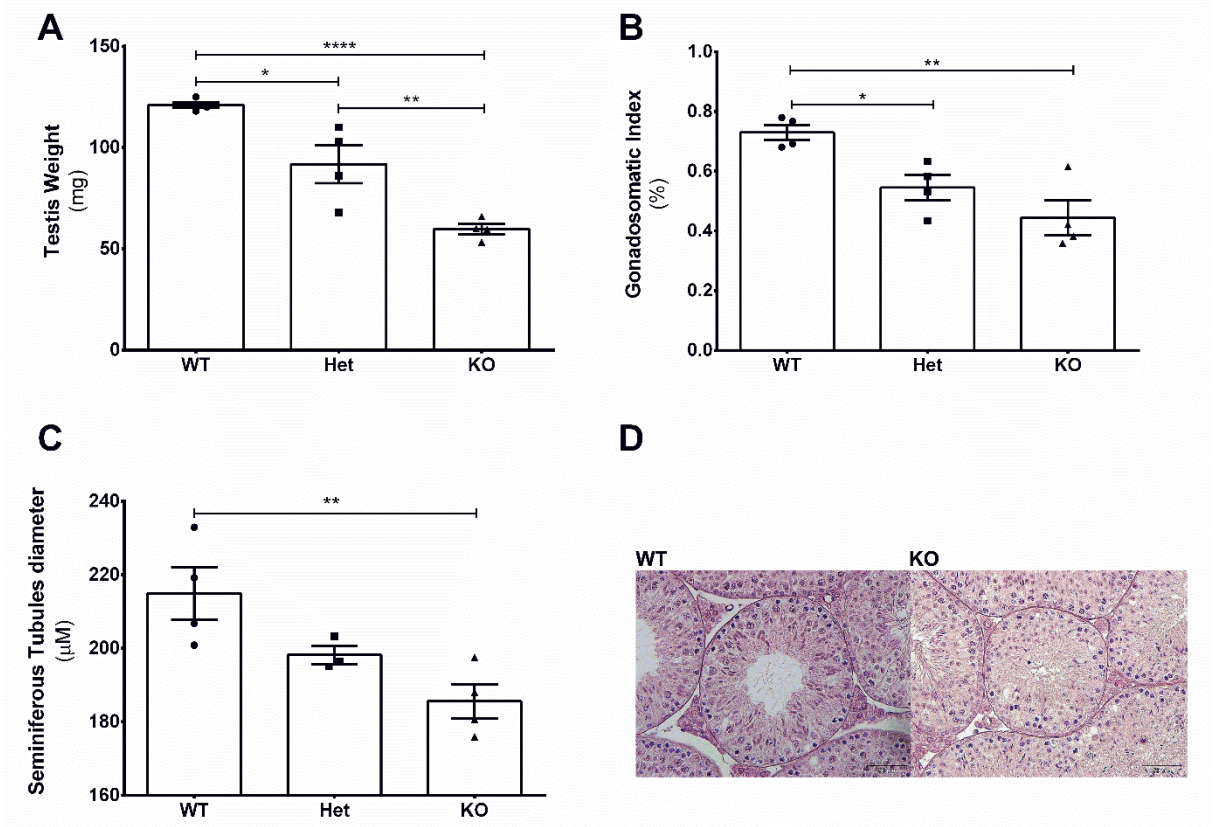

Figure 28 – Effect of insulin-degrading enzyme on testicular morphology. The figure shows data of testis weight (panel A), gonadosomatic index (panel B), seminiferous tubules diameter (panel C) and representative images from seminiferous tubules of wild type (WT) and knockout (KO) mice (panel D) for *Ide*. Results are expressed as mean ± SEM (n=4 for each condition). Significantly different results are indicated as: * $p<0.05$; ** $p<0.01$; **** $p<0.0001$.

Insulin-degrading enzyme affects mice sperm morphology

As insulin regulation is crucial for spermatogenesis and IDE is the enzyme responsible for insulin inactivation, we hypothesized that IDE could affect sperm quality. Our results showed no differences in sperm motility (Figure 29A) and concentration (Figure 29B) of neither heterozygous nor KO mice for Ide. However, Ide KO mice showed decreased sperm viability (43.17 ± 2.18%) when compared to the WT (55.00 ± 2.74%; Figure 29C). Moreover, abnormal sperm morphology was increased in KO (60.00 ± 2.73%) when compared to WT (43.50 ± 2.63%) and Het (37.83 ± 5.44%; Figure 29D).

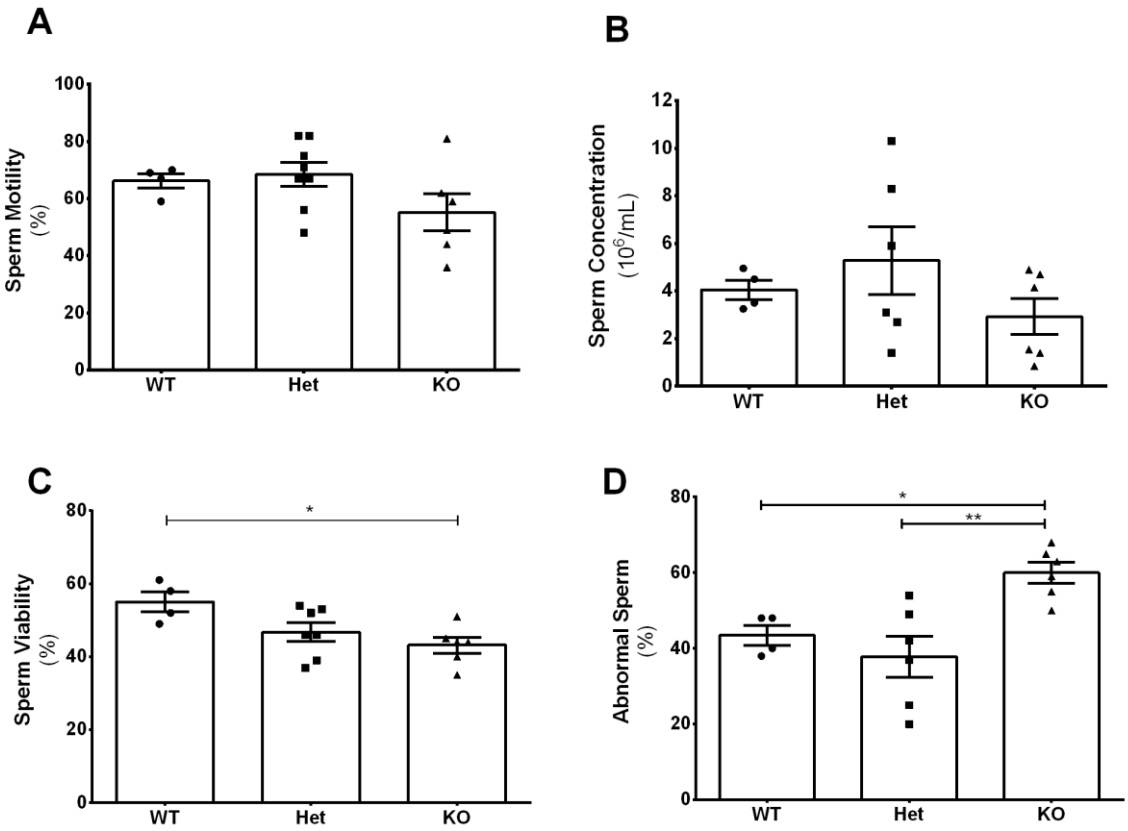

Figure 29 – Effect of insulin-degrading enzyme on sperm parameters. The figure shows data of sperm motility (panel A), concentration (panel B), viability (panel C) and morphology (panel D) from heterozygous (Het) or knockout (KO) mice for *Ide* and wild type (WT) mice. Results are expressed as mean ± SEM (n=4 for each condition). Significantly different results are indicated as: * $p<0.05$; ** $p<0.01$.

Oxidative stress markers in the testicular tissue of Ide KO mice

Oxidative stress is one of the major causes for decreased sperm quality. It damages lipids and proteins compromising sperm quality. Thus, we evaluated lipid peroxidation, protein nitration and carbonylation in testicular tissue of the different mice. When observing both carbonyl groups and protein nitration levels in testicular tissue of Ide KO and heterozygous mice, our results showed no significant differences when comparing with the WT mice (Figure 30A and B). However, there was an increase in testicular tissue lipid peroxidation of Ide KO mice (1.29 ± 0.04 – fold variation to WT), when compared to both Het (1.03 ± 0.01 – fold variation to WT) and WT mice (1.00 ± 0.02; Figure 30C). As one of the causes for lipid peroxidation is the impairment of oxidative phosphorylation, we measured the levels of mitochondrial complexes (Figure 30D) and a decrease in complex IV in the testes of KO (0.54 ± 0.09 – fold variation to WT) when compared to WT (1.00 ± 0.05) was found.

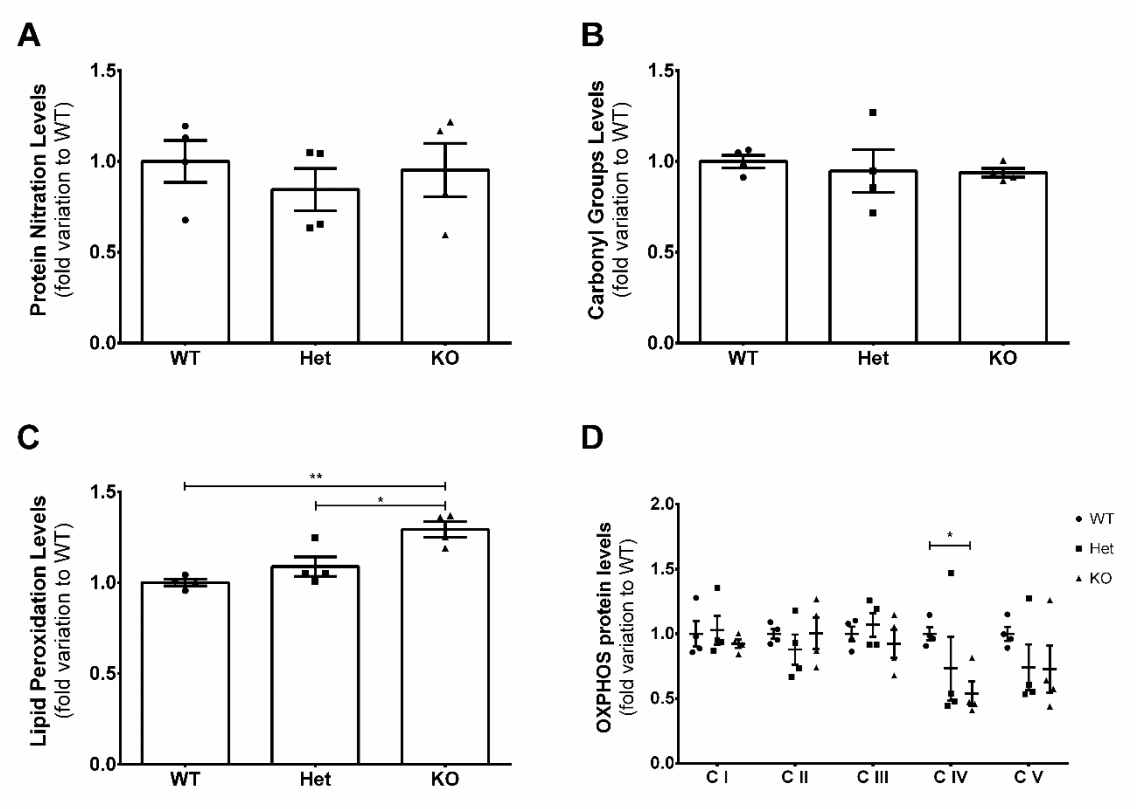

Figure 30 – Effect of insulin-degrading enzyme on oxidative stress markers on testicular tissue. The figure shows data of protein nitration (panel A), carbonyl groups levels (panel B), lipid peroxidation (panel C) and oxidative phosphorylation (OXPHOS) protein levels (panel D) in testicular tissue from heterozygous (Het) or knockout (KO) mice for *Ide* and wild type (WT) mice. Results are expressed as mean ± SEM (n=4 for each condition). Significantly different results are indicated as: * $p<0.05$; ** $p<0.01$.

Apoptotic markers in the testicular tissue of Ide KO mice

Oxidative stress plays a key role in apoptosis thus we analyzed the expression of some key players of this programmed cell death mechanism in the testicular tissue of mice. There was an increase of *Bax* mRNA expression levels in the testicular tissue of *Ide* KO mice (2.43 ± 0.44), when compared to both WT (0.57 ± 0.25) and Het (0.83 ± 0.47) mice (Figure 31A). However, when analyzing both *Bcl2* (Figure 31B) and *Casp3* mRNA levels (Figure 31C), no significant differences were found between the groups.

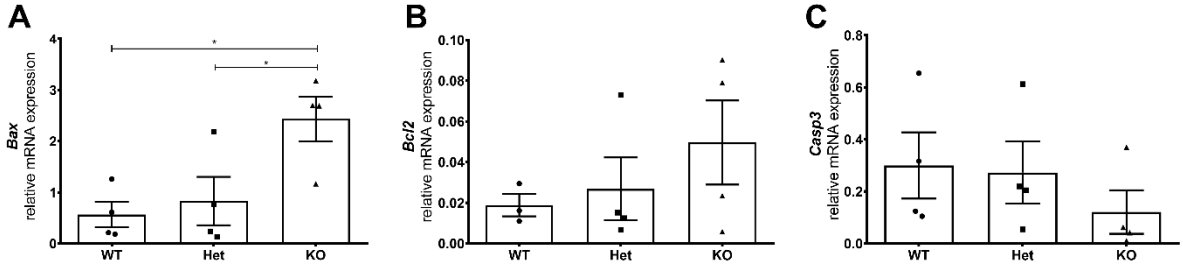

Figure 31 – Effect of insulin-degrading enzyme on apoptotic markers in the testicular tissue of mice. The figure shows data of Bax (panel A), Bcl2 (panel B) and Casp3 (panel C) mRNA expression levels in the testicular tissue from heterozygous (Het) or knockout (KO) mice for Ide and wild type (WT) mice. Results are expressed as mean ± SEM (n = 4 for each condition). Significantly different results are indicated as: * $p<0.05$.

Discussion

The prevalence of prediabetes/diabetes in men in reproductive age has been increasing (Chen *et al.*, 2012). Several reports discussed the impact of prediabetes and DM in male fertility and concluded that dysregulated insulin is a key factor for this connection (Alves *et al.*, 2013, Alves *et al.*, 2012, Rato *et al.*, 2016). IDE is a metallopeptidase that degrades and inactivates insulin and several other peptides, thus having a crucial role in insulin clearance (Pivovarova *et al.*, 2013). Although it is already known that insulin dysregulation (hypo- and hyperinsulinemia), affects male reproductive potential (Alves *et al.*, 2012), the role of IDE on male reproductive tract remains completely unknown. Our data shows that IDE affects testicular morphology. In fact, heterozygous mice for *Ide* showed decreased testes weight, that was further noticed in KO mice. Concomitantly, the GSI was decreased in KO animals, illustrating that *Ide* KO mice have testicular atrophy and morphological alterations when compared to the testes of the WT group. However, only KO mice showed decreased seminiferous tubules diameter. Testes weight, along with seminiferous tubules diameter, are largely dependent on the mass and quantity of the different sperm cells, and are associated with compromised spermatogenesis (Rossi and Aeschlimann, 1982). Several studies revealed decreased testes weight and seminiferous tubules diameter in animal models of DM (Cai *et al.*, 2000, Frenkel *et al.*, 1978, Rossi and Aeschlimann, 1982). Our results suggest that IDE is pivotal for a normal testicular weight and seminiferous tubule structure and may be involved in the mechanisms by which individuals with prediabetes and DM present subfertility/infertility.

Reproductive dysfunction and abnormal sperm production is well documented in men suffering with metabolic diseases, namely DM (Vignon *et al.*, 1991), and infertility/subfertility is a common characteristic of men with diabetes. However, most studies are focused on the effects of hyperglycemia rather than the effects of hyperinsulinemia (Agbaje *et al.*, 2007, Du Plessis *et al.*, 2010). The KO mice for *Ide* present hyperinsulinemia, which is an age-dependent feature of this model (Abdul-Hay *et al.*, 2011), and defective sperm parameters that may be a consequence of the detected defective testicular morphology. Although sperm concentration and motility remained similar between the different groups, there were differences in what concerns to sperm viability and morphology. Sperm viability was decreased in KO mice for *Ide*. In addition, sperm morphology defects were also increased in KO mice. Those defects were mostly detected in the head of sperm cells, which normal morphology is very important for processes like its transport through female reproductive tract, capacitation, or even sperm-oocyte fusion (Kot and Handel, 1987). Normal head morphology is associated with higher pregnancy rates and lower miscarriage rates in cases of assisted reproductive techniques, namely intracytoplasmic sperm injection (Bartoov *et al.*, 2003, Berkovitz *et al.*, 2006). Our results point towards a role for IDE in sperm differentiation/maturation. One main cause for abnormal sperm morphology is the unbalance in oxidative status (Gomez *et al.*, 1998, Hosseinzadeh Colagar *et al.*, 2013). Oxidative stress is associated with augmented production of ROS that modify phospholipids and proteins leading to peroxidation and oxidation of thiol groups (Aitken *et al.*, 1989, Hosseinzadeh Colagar *et al.*, 2013). Spermatozoa are particularly susceptible to ROS as they present a significant concentration of PUFA (Koppers *et al.*, 2008) and many possess sensitive double bonds

between carbon atoms that easily undergo non-enzymatic oxidation and fragmentation. In fact, spermatozoa were already found to be affected by endocrine disruptors that cause increased oxidative environment on testes, such as bisphenol A (D'Cruz *et al.*, 2012). Our data shows an increase on testicular tissue lipid peroxidation in *Ide* KO mice when compared to both WT and heterozygous mice for *Ide* illustrating that the testes of these mice have a prooxidant environment. These results illustrate that lack of *Ide* induces an unbalance in oxidative status towards a pro-oxidant status. The major intracellular biological source of ROS is mitochondrial respiration (Aly *et al.*, 2009). We tested this hypothesis and our results show that the increase in lipid peroxidation was not accompanied by dysfunction in total OXPHOS protein illustrating that the pro-oxidant status may be caused by a decrease in antioxidant defenses mediated by lack of IDE rather than an increase in mitochondrial dysfunction (Valko *et al.*, 2007).

The emerging number of men in reproductive age with metabolic diseases, along with an increasing incidence of metabolic disorders in children, adolescents and young adults highlight the need for molecular studies linking these two conditions that must unveil the mechanisms responsible for such connection. Spermatogenesis, the process by which spermatozoa are formed, is a highly regulated process with enormous energetic needs being insulin a crucial player. In fact, while peripheral insulin levels oscillate due to diet and even the circadian rhythm, testes provide a constitutive source of insulin for spermatogenesis (Gomez *et al.*, 2009), in order to minimize these fluctuations. Moreover, Sertoli cells secrete insulin to provide adequate levels of this hormone to germ cells that do not have direct access to plasma insulin due to the blood-testis barrier (Schoeller *et al.*, 2012). Furthermore, the absence of insulin shifts the glycolytic metabolism of Sertoli cells to Krebs cycle, which compromises germ cell development (Alves *et al.*, 2012, Rato *et al.*, 2016). This work illustrates that IDE acts on glucose metabolism beyond liver, in peripheral organs such as testes. Notably, lack of this enzyme induces lower sperm quality with a lower percentage of viable spermatozoa as well as more defective sperm cells. Besides the relevance for the understanding of subfertility/infertility in men suffering with metabolic diseases, we must highlight that most of infertility cases, about 60-75% in men, are idiopathic, which means that the causes remain obscure. Our results show that when IDE, which should be present and functional in all individuals, is lacking or with heterozygous phenotype, fertility problems may arise in men.

V. GENERAL DISCUSSION

The prevalence of metabolic disorders, such as obesity and type 2 diabetes *mellitus*, is exponentially increasing worldwide, being considered two of the 21st century pandemics. These have numerous hallmarks, such as insulin resistance and inflammation. Moreover, these disorders are associated with several co-morbidities, namely non-alcoholic fatty liver disease (NAFLD), reproductive dysfunction, kidney disease, and cardiovascular disease. Considering the socio-economic characteristics and the clinical burden of these pathologies it is essential to investigate the molecular mechanisms involved in the pathogenesis of these diseases. Similarly, the discovery of biomarkers that can better predict the onset and even the treatment response to a given disorder, moving towards personalized medicine, is also key.

The liver is a vital metabolic organ responsible for regulation of energy metabolism. After a meal the liver uptakes glucose and is responsible for converting it into glycogen. In the hepatocyte fatty acids are converted into triglycerides that can either be stored in lipid droplets or exported into the blood stream as very-low density proteins. The absorbed amino acids are metabolized to provide energy or used to synthesize proteins, glucose, or other molecules. During fast, glycogen and triglycerides are metabolized in the liver and released to the circulation to be further metabolized by the muscle and other extra hepatic tissues; adipose tissue releases non-esterified fatty acids and glycerol via lipolysis. Lactate and alanine are generated from the breakdown of glycogen in the muscle. While glycerol, lactate and alanine, will be delivered into the liver to be used has precursors to glucose synthesis, via gluconeogenesis; non–esterified fatty acids will be oxidized in hepatic mitochondria through fatty acid oxidation and generate ketone bodies. Both glucose and ketone bodies will then provide metabolic fuels for extra hepatic tissues.

Several intervenients have already been identified and proved to be involved in controlling glucose, lipid and amino acid metabolism in insulin-sensitive organs; nevertheless, a complete understanding of all molecules putatively involved in the pathogenesis of prediabetes, NAFLD and associated co-morbidities is crucial.

In the first part of this thesis the aim was to understand the impact of a western diet (high-fat, high-sucrose) and a high-fructose diet on whole-body homeostasis and, particularly, on the metabolome of insulin-sensitive organs, as the liver, skeletal muscle and brown adipose tissue. In C57Bl6/J mice subjected to a 12-week period of a high-fat or high-fructose feeding, the effect of these diets on whole-body homeostasis was examined. While high-fat fed mice developed hyperglycemia, glucose intolerance and insulin resistance, the effect of high-fructose diet on glucose homeostasis was equivalent to the control. In fact, although fructose is reported as being highly lipogenic and implicated in insulin resistance and hepatic steatosis (Jahn *et al.*, 2019, Jegatheesan and De Bandt, 2017), in the present study only a mild effect on overall insulin sensitivity was observed. However, when analyzing hepatic insulin signaling, both high-fat and high-fructose diet decreased insulin sensitivity through a decrease in Akt phosphorylation. Moreover, HFat mice developed NAFLD whereas HFruct mice already presented mild hepatic degeneration in periportal areas. Taken together, these data indicate that both diets impaired insulin sensitivity, although in HFruct these results were independent of obesity. We hypothesized that high-fat and high-fructose diets have a different impact

on the metabolome of insulin-sensitive tissues. In the liver, and through a principal component analysis, we observed that HFruct mice present a metabolome more similar to the Chow than HFat This might be due to the fact that HFat diet is a combination of lipids and sucrose while HFruct diet has only an enrichment of fructose with no alterations in lipid intake, when compared to the Chow diet. However, one common effect of both diets was the depletion of glycogen stores. In fact, glucose metabolism seems to be severely affected in HFat mice, as glucose levels are also decreased, and glucokinase is increased, suggesting an adaptive mechanism to keep glucose trapped inside the hepatocyte. Moreover, HFat mice have increased levels of glycine and taurine. Being these essential components of bile acids and taking into account that cholesterol was also increased in these mice, these results point towards an increased synthesis of bile acids, probably as a result of the increased dietary lipid content. Glycerol was also increased in HFat mice, indicating that adipose tissue lipolysis may be increased. These results are in accordance with previous studies demonstrating that adipose tissue synthesizes and secretes glycerol in cases of high glucose levels (Rotondo *et al.*, 2017). Besides the liver, the skeletal muscle is an essential organ for glucose homeostasis, as it is responsible for glucose uptake, metabolism and disposal. In concurrence with the results obtained in the liver, glucose metabolism was also affected in the muscle of HFat fed mice. In fact, glucose and lactate levels were decreased in HFat mice, thus muscle is using other substrates to obtain energy. Valine, from which ammonia byproducts are used in the conversion of glutamate to glutamine, were decreased in the muscle of both HFat and HFruct. Thus, more ammonia was available for the conversion of glutamate into glutamine, that was increased in the muscle of HFat mice. This points towards an increased transport of glutamine to the liver. In fact, valine levels were already positively associated with insulin resistance (Tai *et al.*, 2010). Once more, HFat triggered further changes in metabolome than HFruct. On the other hand, when BAT metabolome was analyzed, HFruct had more impact than HFat, which gives new insights about the target organs of a high-fructose diet.

HFat led to a decrease in GSH in BAT. GSH was found to be inversely correlated with the activation of thermogenesis in white adipocytes. The inhibition of GSH led to the activation of forkhead box O1 and consequently to the activation of uncoupling protein 1 (Lettieri Barbato *et al.*, 2015), thus the decrease in GSH might be increasing thermogenesis in HFat mice (Mercer and Trayhurn, 1984). Moreover, HFat had increased levels of acetate, that upregulates mitochondrial biogenesis, which is the key organelle for thermogenesis (Hu *et al.*, 2016). On the other hand, HFruct led to an increase in betaine that was reported to protect against fructose-induced inflammation in astrocytes (Li *et al.*, 2015). HFruct also presented increased leucine, which is an activator of the mammalian target of rapamycin (mTOR) pathway, leading to reduced thermogenesis (Cheng *et al.*, 2010, Wang *et al.*, 2016). Thus, HFat seems to be an activator of thermogenesis, while HFruct downregulates it.

High-fat and high-fructose diets are reported to cause increased oxidative stress and a state of chronic inflammation. Knowing the impact of these diets on the liver, the levels of PON1, an important antioxidant and inflammatory enzyme, were analyzed. Indeed, although synthesized in the liver, PON1 has a systemic role, as it is the responsible for HDL antioxidant functions. Both HFat and HFruct induced a decrease of hepatic PON1 protein levels, although HFat promoted lower PON1 levels than

HFruct. This indicated that PON1 is being less secreted and thus, HDL might be less effective in counteracting LDL oxidation, thus contributing for the pro-inflammatory environment.

With these results, we then hypothesized that PON1 has a role in protecting against ectopic lipid deposition and insulin resistance. Thus, we proceeded to the evaluation of the impact of these diets on a *Pon1* whole-body knockout (KO) mouse model, after 12 weeks of a normal chow diet (NCD), high-fat (HFat) or high-fructose diet in both wildtype (WT) and KO mice. Although the absence of PON1 did not cause changes in weight gain, it caused glucose intolerance in NCD and HFat mice, implying a role for PON1 in glucose homeostasis. Although HFruct KO mice did not present differences in glucose tolerance compared with HFruct WT mice, even the latter were glucose intolerant. These results indicate that, regarding overall glucose tolerance, PON1 has a more prominent role in a context of normal chow and HFat than HFruct feeding.

Moreover, C-peptide levels were increased in KO mice in the three diets. However, insulin levels were similar between WT and KO. This could indicate and points towards increased insulin clearance. In fact, there is a study mentioning that PON1 has a protective role in ß-cells, stimulating insulin release (Koren-Gluzer *et al.*, 2011), and thus contradictory with our data. However, a second hypothesis arises, that C-peptide degradation is impaired, which indicates that KO mice might have kidney dysfunction (Jaspan *et al.*, 1977). Indeed, another group already reported decreased PON1 activity in kidney dysfunction (Miljkovic *et al.*, 2018).

Given that PON1 levels are decreased in hyperglycemic mice; PON1 KO mice have defects in glucose tolerance; and PON1's atheroprotective role (Aviram and Rosenblat, 2004, Getz *et al.*, 2010, Rosenblat *et al.*, 2005) we further hypothesized that PON1 could be a co-biomarker for dysmetabolism. The recent interest in the search for new biomarkers has grown because metabolic disorders are highly heterogeneous, complex, making the "one size fits all" theory useless (McCarthy, 2017). The motivation for personalized medicine is that individuals who have the same disease may have very heterogeneous metabolic characteristics that are reflected in their health and response to treatment. In the context of metabolic disorders, new biomarkers would help to understand which individuals have a higher risk to develop complications and, importantly, why do they develop them.

To test our hypothesis, serum PON1 paraoxonase activity (POase) was measured in 304 individuals from the PREVADIAB2 study and POase was used along with lipid profile, age, estimated glomerular filtration rate and ApoJ levels in a cluster analysis. This analysis revealed four distinct clusters, with a potentially distinct pathophysiological meaning. One of the clusters grouped young individuals, apparently healthy and mostly normoglycemic, with high POase activity. The second cluster grouped individuals that although older than the first, were younger than the studied population. Most of the subjects presented dysglycemia, dyslipidemia and renal hyperfiltration. Indeed, this cluster, being the most metabolically unhealthy, was also one of the clusters with lower POase activity. The other two clusters were very similar between them, except for POase activity. In fact, these individuals had an intermediate profile compared to the first two groups, in terms of glucose tolerance and insulin secretion. Given the low POase activity without significant dysglycemia and dyslipidemia, two hypotheses arose for this result. On one hand, we hypothesize that these individuals present HDL

dysfunction, from which decreased PON1 activity is characteristic. This HDL dysfunction might be caused by kidney disease (Kaseda *et al.*, 2015, Rysz *et al.*, 2020), which goes in line with our results in KO mice. Moreover, we also hypothesized that these individuals may have PON1 polymorphisms that are known to cause decreased PON1 activity or decreased PON1 levels, that will then cause a decrease in PON1 activity (Hatzihidiroglou *et al.*, 2016). Thus, PON1 may actually be used as a co-biomarker of dysmetabolism.

Metabolic disorders and, specifically insulin and glucose dysregulations, cause impairments in almost every organ (Buysschaert *et al.*, 2015). The increasing number of men in reproductive age with metabolic diseases, along with a rising incidence of metabolic disorders in children, adolescents and young adults (Delfino *et al.*, 2007) highlight the need for studies linking these two conditions to expose the mechanisms responsible for such connection. Within the seminiferous tubules, proper insulin and glucose metabolism are crucial for male reproductive function, since several essential metabolites for developing germ cells are produced from glucose (Rato *et al.*, 2016). Moreover, developing germ cells are highly susceptible to inflammation and oxidative stress, hallmarks of metabolic disorders, due to their low antioxidant defenses and high percentage of PUFA (Lenzi *et al.*, 1994). As most of the studies analyzing (in)fertility in metabolic disorders are related with hyperglycemia, we aimed to address the effects of primary hyperinsulinemia on male reproductive function. To test the hypothesis that insulin-degrading enzyme is a key player in insulin dysfunction-associated male infertility, we used an *Ide* whole-body KO model. The lack of this enzyme induced morphological changes in the testes and decreased percentage of viable spermatozoa. There was also an increase in defective sperm cells, which might impair fertilization (Bartoov *et al.*, 2003). Moreover, the absence of IDE caused a proinflammatory environment, illustrated by increased lipid peroxidation in testicular tissue. This work highlighted that IDE influences glucose and insulin metabolism beyond liver, in peripheral organs such as testes.

There are several proteins and mechanisms involved in the pathogenesis of prediabetes and its co-morbidities. The studies included in this dissertation contribute for a further knowledge on the impact of high-fat and high-fructose diets on insulin-sensitive organs. To our knowledge, this is the first study evaluating the metabolome of important metabolic organs (liver, muscle and brown adipose tissue) upon high-fat or high-fructose diet. Here, we could observe the distinct effects caused by these diets and how the tissue crosstalk. Moreover, the role of PON1 and IDE on glucose and insulin metabolism was unveiled. PON1 is involved in glucose tolerance and might be used as a co-biomarker with currently clinically available parameters to allow a refinement of dysmetabolism diagnosis and prognosis. However, new hypotheses arose about the involvement of PON1 in kidney disease. On the other hand, IDE, by avoiding hyperinsulinemia, is essential for the maintenance of male reproductive function. Together, these data contribute to a better understanding of the pathophysiological events involved in prediabetes and two of its comorbidities: non-alcoholic fatty liver disease and male reproductive dysfunction.

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VII. ANNEXES

Annex I – Chow Diet Datasheet

Rat and Mouse No.3 Breeding (E)

Calculated Analysis

NUTRIENTS		Total	Supp (9)
Proximate Analysis			
Moisture (1)	%	10.00	
Crude Oil	%	4.25	
Crude Protein	%	22.39	
Crude Fibre	%	4.21	
Ash	%	7.56	
Nitrogen Free Extract	%	51.20	
Digestibility Co-Efficients (7)			
Digestible Crude Oil	%	3.86	
Digestible Crude Protein	%	20.21	
Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)			
Total Dietary Fibre	%	15.43	
Pectin	%	1.43	
Hemicellulose	%	9.20	
Cellulose	%	3.93	
Lignin	%	1.50	
Starch	%	33.92	
Sugar	%	5.75	
Energy (5)			
Gross Energy	MJ/kg	15.21	
Digestible Energy (15)	MJ/kg	12.42	
Metabolisable Energy (15)	MJ/kg	11.36	
Atwater Fuel Energy (AFE) (8)	MJ/kg	13.90	
AFE from Oil	%	11.50	
AFE from Protein	%	26.93	
AFE from Carbohydrate	%	61.57	
Fatty Acids			
Saturated Fatty Acids			
C12:0 Lauric	%	0.05	
C14:0 Myristic	%	0.20	
C16:0 Palmitic	%	0.36	
C18:0 Stearic	%	0.09	
Monounsaturated Fatty Acids			
C14:1 Myristoleic	%	0.01	
C16:1 Palmitoleic	%	0.13	
C18:1 Oleic	%	1.03	
Polyunsaturated Fatty Acids			
C18:2(ω6) Linoleic	%	1.15	
C18:3(ω3) Linolenic	%	0.17	
C20:4(ω6) Arachidonic	%	0.22	
C22:5(ω3) Clupanodonic	%	0.04	
Amino Acids			
Arginine	%	1.54	
Lysine (6)	%	1.33	0.09
Methionine	%	0.34	
Cystine	%	0.34	
Tryptophan	%	0.27	
Histidine	%	0.57	
Threonine	%	0.86	
Isoleucine	%	0.98	
Leucine	%	1.68	
Phenylalanine	%	1.03	
Valine	%	1.10	
Tyrosine	%	0.80	
Taurine	%		
Glycine	%	1.88	
Aspartic Acid	%	1.43	

NUTRIENTS		Total	Supp (9)
Glutamic Acid	%	4.07	
Proline	%	1.38	
Serine	%	0.97	
Hydroxyproline	%	0.06	
Hydroxylysine	%		
Alanine	%	0.14	
Macro Minerals			
Calcium	%	1.15	0.56
Total Phosphorus	%	0.82	0.09
Phytate Phosphorus	%	0.25	
Available Phosphorus	%	0.58	0.09
Sodium	%	0.32	0.19
Chloride	%	0.43	0.31
Potassium	%	0.81	
Magnesium	%	0.29	0.04
Micro Minerals			
Iron	mg/kg	188.17	82.50
Copper	mg/kg	20.28	8.75
Manganese	mg/kg	102.01	52.70
Zinc	mg/kg	51.34	8.64
Cobalt	µg/kg	617.02	525.00
Iodine	µg/kg	1395.12	775.00
Selenium	µg/kg	497.70	200.00
Fluorine	mg/kg	9.24	
Vitamins			
β-Carotene (2)	mg/kg	0.15	
Retinol (2)	µg/kg	5977.24	5812.50
Vitamin A (2)	iu/kg	19923.60	19375.00
Cholecalciferol (3)	µg/kg	102.22	72.50
Vitamin D (3)	iu/kg	4088.65	2900.00
α-Tocopherol (4)	mg/kg	100.35	81.14
Vitamin E (4)	iu/kg	110.39	89.25
Vitamin B_1 (Thiamine)	mg/kg	27.08	19.11
Vitamin B_2 (Riboflavin)	mg/kg	10.60	7.60
Vitamin B_6 (Pyridoxine)	mg/kg	19.54	14.46
Vitamin B_{12} (Cyanocobalamine)	µg/kg	26.78	17.75
Vitamin C (Ascorbic Acid)	mg/kg	1.33	
Vitamin K (Menadione)	mg/kg	4.15	3.72
Folic Acid (Vitamin B_9)	mg/kg	2.73	0.49
Nicotinic Acid (Vitamin PP) (6)	mg/kg	85.00	19.11
Pantothenic Acid (Vitamin $B_{3/5}$)	mg/kg	40.27	23.80
Choline (Vitamin $B_{4/7}$)	mg/kg	1641.65	366.60
Inositol	mg/kg	1903.20	
Biotin (Vitamin H) (6)	µg/kg	322.87	

Notes

1. All values are calculated using a moisture basis of 10%. Typical moisture levels will range between 9.5 - 11.5%.
2. a. Vitamin A includes Retinol and the Retinol equivalents of β-carotene
   b. Retinol includes the Retinol equivalents of β-Carotene.
   c. 0.48 µg Retinol = 1 µg β-carotene = 1.6 iu Vitamin A activity
   d. 1 µg Retinol = 3.33* iu Vitamin A activity
   e. 1 iu Vitamin A = 0.3 µg Retinol = 0.6 µg β-carotene
   f. The standard analysis for Vitamin A does not detect β-carotene
3. 1µg Cholecalciferol (D_3) = 40.0 iu Vitamin D
4. 1 mg all-*rac*-α-tocopherol = 1.1 iu Vitamin E activity
   1 mg all-*rac*-α-tocopherol acetate = 1.0 iu Vitamin E activity
5. 1 MJ = 239.23 Kcalories = 239.23 Calories = 239,230 calories
6. These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
7. Based on in-vitro digestibility analysis.
8. AF Energy = Atwater Fuel Energy = ((CO%/100)*9000)+((CP%/100)*4000)+((NFE%/100)*4000)/239.23
9. Supplemented nutrients from manufactured and mined sources.
15. Calculated.

Figure 32 – Composition of the Normal Chow Diet. Composition of the Normal Chow Diet (RM-3, SDS Diets) used in the studies with C57Bl6/J and B6.129X1-*Pon1*^{tm1Lus}/J mice included in this thesis (Special Diets Services).

Annex II – High-Fat Diet Datasheet

Open formula purified diets for lab animals

OpenSource DIETS®

Product Data - D12331

Report ▸ Repeat ▸ Revise

Description
58 kcal% fat w/sucrose
Surwit Diet

Used in Research
Obesity
Diabetes

Packaging
Product is packed in 12.5 kg box. Each box is identified with the product name, description, lot number and expiration date.

Lead Time
IN-STOCK.
Ready for next day shipment.

Gamma-Irradiation
Yes. Add 10 days to delivery time.

Form
Pellet, Powder

Shelf Life
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months. Because of the high fat content is best if kept frozen.

Control Diets
D12329

Special Considerations
Contains no fiber.
High in sodium.

Formula

Product #D12331		gm%	kcal%
Protein		23.0	16.4
Carbohydrate		35.5	25.5
Fat		35.8	58.0
	Total		100
	kcal/gm	5.56	
Ingredient		**gm**	**kcal**
Casein, 30 Mesh		228	912
DL-Methionine		2	0
Maltodextrin 10		170	680
Corn Starch		0	0
Sucrose		175	700
Soybean Oil		25	225
Coconut Oil, Hydrogenated		333.5	3001.5
Mineral Mix S10001		40	0
Sodium Bicarbonate		10.5	0
Potassium Citrate, 1 H2O		4	0
Vitamin Mix V10001		10	40
Choline Bitartrate		2	0
FD&C Red Dye #4		0.1	0
Total		**1000.1**	**5558.5**

Professor Richard Surwit designed these diets with us for his diet-induced obesity studies at Duke University.
Diets match 10/27/92 telephone specifications of R. Surwit, Ph. D., Duke University. Formulated by E. A. Ulman, Ph.D., Research Diets, Inc. November 6, 1992.

RESEARCH DIETS INC.
www.ResearchDiets.com
Where NutriPhenomics Begins

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901 USA
Tel: 732.247.2390
Fax: 732.247.2340
info@researchdiets.com

Figure 33 – Composition of the High-Fat Diet. Composition of the High-Fat Diet (D12331, Research Diets) used in the studies with mice included in this thesis (Research Diets Inc.).

Annex III – Supplementary Data

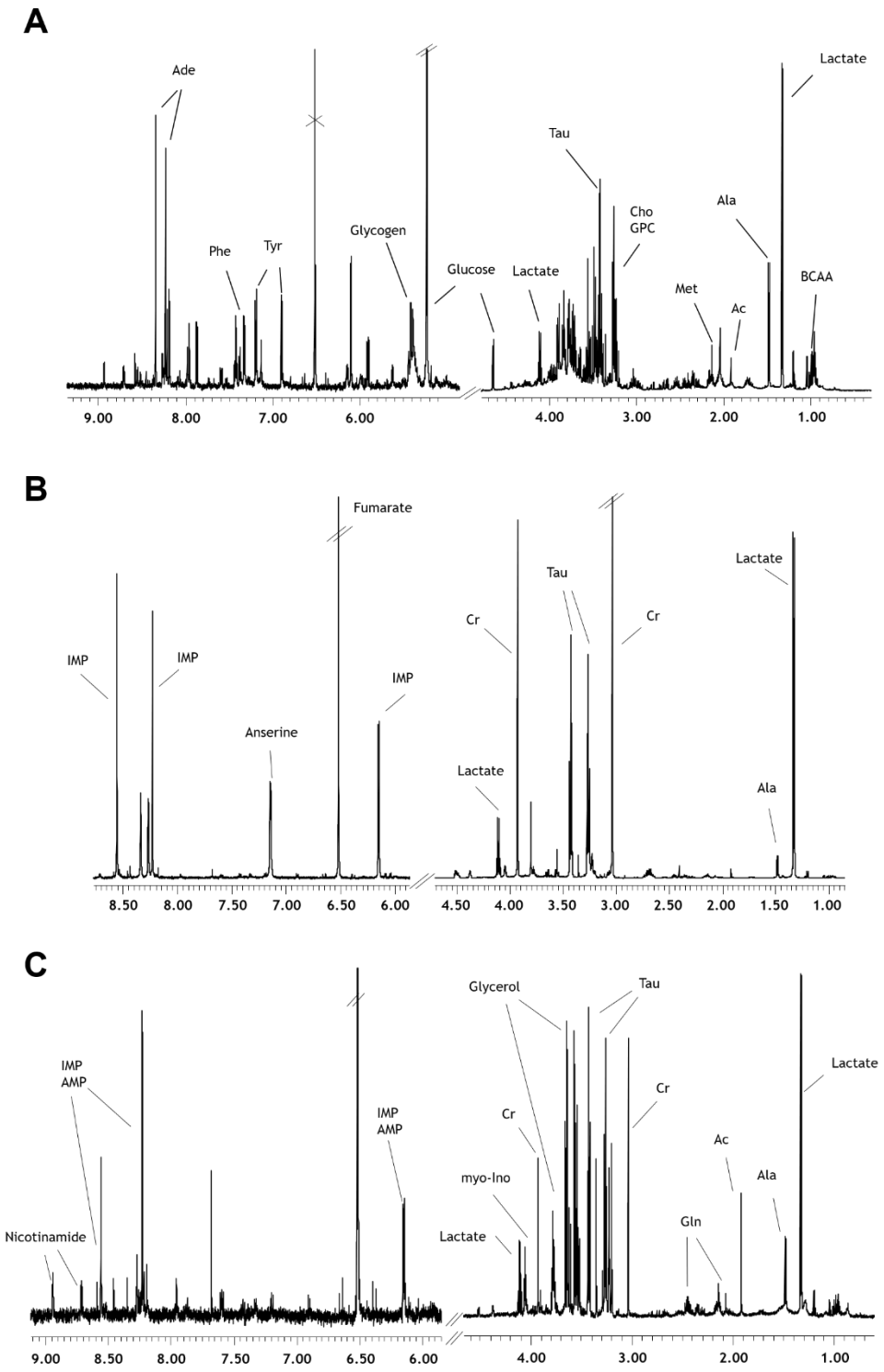

Figure 34 – Typical 1D NMR spectra of hepatic, muscle and brown adipose tissue polar extracts. A - Typical 1D NMR spectra of hepatic polar extracts with low field region (5.2-9.5 ppm) vertically expanded. B - Typical 1D NMR spectra of muscle polar extracts with low field region (5.2-9.0 ppm) vertically expanded. C - Typical 1D NMR spectra of BAT polar extracts with low field region (5.2-9.2 ppm) vertically expanded. Ac – acetate; Ade – adenine; BCAA - branched chain amino acids; Cho – choline; Cr – creatine; GPC – glycerophosphocholine; IMP - inosine monophosphate; myo-Ino - myo-inositol; Tau - taurine; three letter code used for amino acids.

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