The pathophysiological role of physical inactivity and fat-diet to development of metabolic syndrome in an animal model

Academic thesis with the purpose of obtaining a doctoral degree in Physical Activity and Health under the law 74/2006 from March 24th

**Supervisor**: José Alberto Ramos Duarte, MD, PhD

**Co-supervisor**: Maria do Amparo Andrade, PhD

**José Antonio Franchi Bovolini**

Porto, 2019

Keywords: Physical inactivity, fat diet, metabolic syndrome, liver disorder, and endocrine pancreas disarrangement.
The candidate was supported by a joint fellow from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/BEX 0807/14-1), a foundation from Brazilian Ministry of Education (MEC).

The experimental studies presented in this thesis were supported and performed at the Research Center for Physical Activity, Health and Leisure (CIAFEL), housed in the Sports Faculty of University of Porto (FADEUP), Porto, Portugal.
This work is dedicated to my parents,

José Antonio Bovolini e Helenita Franchi Bovolini.
São tantas as pessoas que eu gostaria de agradecer que seriam necessárias outras cem páginas para que eu pudesse citar a todos que fizeram parte deste trabalho e outras tantas que fizeram parte de uma jornada que culmina com esta tese. Uma jornada incrível onde ganhei novas perspetivas do mundo e principalmente de mim mesmo! Vocês farão sempre parte da minha vida, muito obrigado.

Primeiramente, eu gostaria de agradecer ao meu orientador Prof. Dr. José Alberto Ramos Duarte por me receber durante tantos anos no laboratório de bioquímica do desporto e de morfologia experimental. Obrigado por abrir as portas de mundo novo onde aprendi mais do que eu achei que seria capaz. No entanto, a inexperiência parece ser uma fiel companheira dos novos desafios e por isso agradeço pela sua paciência. Obrigado principalmente pelos momentos de aprendizado e pelo tempo que me foi dedicado ao longo de tantos anos. O seu brilhantismo, sabedoria e capacidade de trabalho serão sempre uma referência para mim. Espero ter sido um bom aluno. Muito obrigado pela oportunidade em aprender consigo.

O meu mais sincero agradecimento à minha coorientadora Prof.ª Dr.ª Maria do Amparo Andrade. Obrigado por aceitar a tarefa de guiar meu caminho académico e pelo exemplo constante de trabalho. Obrigado pelo companheirismo, amizade e inabalável alegria nas incontáveis horas de trabalho laboratorial. Agradeço sobretudo pelo seu exemplo de perseverança ao longo desta tarefa. Eu levarei sempre comigo a admiração pela sua história pessoal e profissional. A senhora será sempre um grande exemplo para minha carreira.

À Celeste Maria Resende dos Santos, a minha “Celestinha”. Foram tantos anos a trabalhar juntos que a amizade foi inevitável. Obrigado pelo apoio incondicional, pelo cuidado comigo e com meu trabalho e por tantos momentos partilhados. Obrigado pelas intermináveis láminas cortadas, por tantas gaiolas lavadas e por tantos ratos pesados. Obrigado pelas conversas partilhadas junto
ao micrótomo, à beira da mesa ou no calor da churrasqueira. Longe de casa, o abraço da senhora foi uma âncora nos difíceis dias que passei. Muito obrigado.


Ao amigo Daniel Moreira-Gonçalves. Obrigado pela amizade, pelo conhecimento e pelo exemplo de integridade, dedicação e ética. Tenho orgulho em aprender e de ter a si como amigo. À Nádia Gonçalves e Ana Filipa Silva. Obrigado pela amizade, pelo exemplo de dedicação e por toda ajuda que me foi dedicada. A vossa contribuição foi fundamental no meu percurso. Sinto-me honrado de tê-las como amigas e por ter a vossa marca no meu trabalho. Muito obrigado por tudo.

Ao Prof. José Oliveira pelo suporte e por tantos abraços amigáveis. Em dias nublados, um abraço sincero torna-se um farol. Ao grande Prof. Jorge Mota pelo suporte e pelas palavras de incentivo. À Sra. Maria Domingues por toda a ajuda, pela amizade e pelo cuidado durante esses anos todos. Muito obrigado pelo sorriso fácil e pela paciência. Aos amigos David Rizo e Estela Alves pelos ensinamentos, amizade e por tantos socorros nos momentos de dúvida. À Inês Aleixo, obrigado pelo exemplo de amor e dedicação à academia. Muito obrigado pelas conversas, pela ajuda e pela amizade. Admiro-te imenso. À Lucimere Bohn pela amizade, pelo incentivo constante e pelo exemplo de resiliência. Eu sinceramente espero que colhas os frutos da tua dedicação.

À Juliana Garcia, obrigado pela amizade e pelo exemplo de determinação e competência. Não tenho palavras para expressar a minha
gratidão por toda a sua ajuda, pelas palavras constantes de incentivo e por reconhecer minha dedicação a este trabalho. Você sempre será uma referência de competência e resiliência. O meu mais sincero obrigado. Você foi fundamental na minha jornada.

Aos amigos de longa data Renata Willig, Cesar Agostinis e Cristine Schmidt. Foi incrível percorrer esse longo caminho com vocês! Muito obrigado por tantos anos de amizade e suporte. ESPero tê-los sempre por perto e que a vida nos mantenha sempre próximos. Rê, obrigado pelo exemplo de força e determinação e por estender a mão nos momentos que mais precisei. Obrigado pelo companheirismo e pela amizade incondicional! Cris, obrigado por estar ao meu lado nos momentos mais importantes, pelo apoio e principalmente pela amizade. Cesão, obrigado pelos anos de companheirismo e pelo exemplo de perseverança e trabalho. Vocês farão sempre parte da minha história.

Ao meu amigo Ivo Garcia, pela amizade quando mais precisei, pelo constante encorajamento e principalmente por partilhar comigo o amor pelos “azuis” e por este país. Ao Lucas Monteiro, pelo suporte constante e amizade.

Ao meu grande amigo Giorjines Boppre pelo companheirismo e amizade sincera. Obrigado pelo incentivo constante e por não me deixar esquecer que não há nada mais nobre que a amizade. À Joana Ribeiro, uma amizade que leverei dos corredores da FADEUP para a vida. Obrigado pelas longas conversas, por partilharmos tantos momentos e pela amizade que construímos.

À Rose Autran, o meu exemplo maior de superação e de resiliência humana. Aprendi tanto contigo! Muito obrigado pelo apoio constante e por estar ao meu lado nos dias de escuridão. Tenho um bem-querer do tamanho do Rio Grande por ti! Ao meu amigo Nelson Knak, pelas conversas acompanhadas pelo chimarrão e pela mais honesta das amizades. Obrigado por confiarem em mim e por estarem ao meu lado neste capítulo importante da minha vida. Tenho muito orgulho da nossa amizade.

À Sara Tribuzi pelo cuidado minucioso com cada vírgula escrita neste trabalho, por me fazer pensar sobre tantas e tantas coisas, mas principalmente pela amizade sincera. Admiro e gosto muito de ti. Ao meu amigo Márcio
Borgonovo, pelo exemplo de superação, pelo incentivo constante e por ser a prova que ser fiel ao nosso sonho é o caminho a seguir.

À Diana Ospina, obrigado por tudo. Obrigado pela amizade incondicional, pelo companheirismo, pelo cuidado constante e por apenas partilhares comigo sinceridade. Obrigado pelo exemplo de bondade e doação. Tua amizade enche o meu peito de orgulho e de admiração.

À CAPES pela oportunitade em realizar o doutorado fora do país.

E finalmente à minha família. Aos meus avós Ovídio e Iva Bovolini, e Luís Paulo e Maria Franchi. Obrigado pelo exemplo de perseverança e por cultivar o orgulho das nossas origens. Espero que estejam orgulhosos. Aos meus cunhados Milene Bovolini (meu exemplo de dedicação e determinação) e Wesley Arrussul pela amizade, pelo carinho e cuidado com meus irmãos e meus pais. Fico muito feliz por vocês fazerem parte da nossa família.

Aos meus irmãos Rafael e Rafaela Franchi Bovolini. Vocês foram sempre grande parte da minha saudade, mas também do meu orgulho. Orgulho dos profissionais obstinados e competentes, mas principalmente dos seres humanos em que se tornaram ao longo da minha distância. Obrigado por acreditarem em mim, pela amizade que construímos além dos laços sanguíneos e pelo exemplo de dedicação e de amor. Desculpa pelos momentos importantes da vossa vida em que não pude estar presente.

Aos meus pais José Antonio Bovolini e Helenita Franchi Bovolini. Pai e mãe, obrigado pelo maior exemplo de trabalho, sacrifício e dedicação que alguém poderia ter. Vocês foram sempre o exemplo de honestidade e hombridade que segui durante meu percurso longe de casa. Obrigado pelo apoio incondicional e por me fazerem acreditar que o trabalho e a dedicação ainda são fundamentais. Obrigado por tudo e espero deixá-los orgulhosos. Este trabalho é dedicado a vocês.
# TABLE OF CONTENTS

FUNDING SOURCES ........................................................................................................... V

TABLE OF CONTENTS ...................................................................................................... XIII

LIST OF FIGURES ........................................................................................................ XV

LIST OF TABLES ........................................................................................................... XXI

LIST OF ABBREVIATIONS ........................................................................................... XXIII

RESUMO .......................................................................................................................... XXV

ABSTRACT ...................................................................................................................... XXVII

CHAPTER I · GENERAL INTRODUCTION AND AIMS ..................................................... 1
  General Introduction .......................................................................................................... 3
  Aims ................................................................................................................................. 9

CHAPTER II · STATE-OF-ART ......................................................................................... 11
  Review Article · Metabolic Syndrome: The Pathophysiologic Role of Diet and Sedentary Lifestyle ......................................................................................................................... 13

CHAPTER III · ORIGINAL STUDIES .............................................................................. 51
Study 1 ∙ Influence of diet and physical activity levels, applied from the early stages of growth and development, on the adult phenotype of Wistar rats........................................................................................................ 53

Study 2 ∙ Relative role of fat diet and physical inactivity to metabolic syndrome and non-alcoholic fatty liver disease development in Wistar rats........................................................................................................ 79

Study 3 ∙ Langerhans islets phenotype alterations induced by fat diet and physical activity levels in Wistar rats....................................................................................................................... 109

CHAPTER IV ∙ GENERAL DISCUSSION ............................................................................... 131
Discussion of Methodology................................................................................................. 133
Discussion of Results ......................................................................................................... 139

CHAPTER V ∙ GENERAL CONCLUSIONS ....................................................................... 145

CHAPTER VI ∙ BIBLIOGRAPHY ....................................................................................... 147
LIST OF FIGURES

STUDY I

Figure 1 – Voluntary physical activity weekly recorded on running wheel in AHFD (Active High Fat Diet) and ASD (Active Standard Diet) groups. Data presented as mean ± standard deviation († p<0.0001 vs. ASD) ........................................ 62

Figure 2 – Mean values (± Standard deviation) of total food consumption and total caloric intake are depicted in A and B, respectively, in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups. The mean values variation for each group on food consumption and caloric intake along the experimental protocol are observed in C and D, respectively. (* p<0.05 vs. SSD; *** p<0.001 vs. SSD; **** p<0.0001 vs. SSD; ††† p<0.001 vs. ASD; ‡‡‡‡ p<0.0001 vs. AHFD) ...................... 63

Figure 3 – Mean values (± Standard deviation) of weekly average macronutrients consumption in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups. (*** p<0.001 vs. SSD; **** p<0.0001 vs. SSD; #### p<0.0001 vs. SHFD; †††† p<0.0001 vs. ASD; ‡‡‡ p<0.001 vs. AHFD; ‡‡‡‡ p<0.0001 vs. AHFD) .................................................................................................................. 64

Figure 4 – Variation of body weight mean values along the experimental protocol in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups ................................. 65
**Figure 5** – Mean values (±Standard deviation) of percentage of body mass variation (BMV, %) in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups. (* p<0.05 vs. SSD; ** p<0.01 vs. SSD; # p<0.05 vs. SHFD; † p<0.05 vs. ASD; ‡‡ p<0.01 vs. AHFD) ................................................................. 66

**STUDY II**

**Figure 1** – Total running distance at the end of experimental protocol (Km) from active standard (ASD) and fat diet-fed (AFD) groups. The results are expressed as means ± standard deviation. δ p<0.0001 vs. AFD ........................................... 91

**Figure 2** – Light micrographs of liver sections stained with haematoxilin-eosin, representative of all studied groups: physically inactive standard diet-fed (SSD, a), active standard diet-fed (ASD, b), physically inactive fat-diet fed (SFD, c), and active fat-diet fed (AFD, d) groups. Macrovesicular (yellow arrows) and microvesicular steatosis (black arrows), as well as ballooned cells (blue arrows) are depicted in c and d. The box-plot graphic of non-alchholic fat liver disease (NAFLD) score is illustrated in e. π p<0.0001 vs. SSD; δ p<0.0001 vs. ASD; ω p<0.0001 vs. AFD ................................................................. 93

**Figure 3** – Hepatic light micrographs, stained with picrosirius red, representative of the studied groups: physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d). The median values (P25-P75 and the extreme values) of hepatic expression of collagen in all groups are depicted in e. π p<0.01 vs. SSD; ππ
p<0.0001 vs. SSD; δ p<0.01 vs. ASD; δδ p<0.0001 vs. ASD; ω p<0.01 vs. AFD

Figure 4 – Light micrographs of liver sections representative of all studied groups, processed with terminal deoxynucleotidyl transferase dUTP nick and labeling method: physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d). Mean values ± standard deviation of nuclei/µm² are depicted in e. Stained hepatocyte nuclei are indicated by yellow arrows. π p<0.01 vs. SSD; δ p<0.0001 vs. ASD; ω p<0.0001 vs. AFD

Figure 5 – Light micrographs of liver sections immunostaining for M1 (CD 68) and M2 (Mannose receptor) macrophage phenotypes, representative of all studied groups: physically inactive standard diet-fed (SSD, a and e), physically inactive fat-diet fed (SFD, b and f), active standard diet-fed (ASD, c and g), and active fat-diet fed (AFD, d and h). Mean values ± standard deviation of cells/µm² of M1 and M2 are depicted in i and j, respectively. π p<0.0001 vs. SSD; δ p<0.01 vs. ASD; δδ p<0.0001 vs. ASD; η p<0.0001 vs. SFD; ω p<0.05 vs. AFD; ωω p<0.0001 vs. AFD

Figure 6 – Light micrographs of liver sections immunostaining for NF-κB, representative of all studied groups: physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d). The median values (25thP-75thP) are depicted in e.
\[ \pi \text{ p}<0.01 \text{ vs. SSD}; \delta \text{ p}<0.05 \text{ vs. ASD}; \delta\delta \text{ p}<0.0001 \text{ vs. ASD}; \omega \text{ p}<0.05 \text{ vs. AFD}; \omega\omega \text{ p}<0.0001 \text{ vs. AFD} \]

**STUDY III**

**Figure 1** – Blood glucose concentrations (mg/dL) during the glucose tolerance test (GTT) and the insulin sensitivity test (GTT) in physically inactive standard diet-fed (SSD, ○), physically inactive fat-diet fed (SFD, ●), active standard diet-fed (ASD, △), and active fat-diet fed (AFD, □) …………………………………………………………………………………… 98

**Figure 2** – Representative light micrographs from pancreas of physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d) stained with hematoxylin-eosin. The yellow arrows identify Langerhans islets. Values of CSA are presented in e as a boxplot graphic. \[ \pi \text{ p}<0.05 \text{ vs. SSD}; \mu \text{ p}<0.05 \text{ vs. ASD}; \delta \text{ p}<0.05 \text{ vs. AFD} \]

**Figure 3** – Representative light micrographs of pancreas stained with picrosirius red from physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d), depicting Langerhans islets. The red staining is indicative of collagen content. Mean values ± standard deviation of collagen content (%) in Langerhans islets of the different groups are presented in e. \[ \mu \text{ p}<0.05 \text{ vs. ASD}; \delta \text{ p}<0.05 \text{ vs. AFD} \]

………………………………………………………………………………………… 123
Figure 4 – Fluorescence micrographs of pancreas sections treated with terminal deoxynucleotidyl transferase dUTP method (TUNEL) representative of physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d) groups, showing positive stained nuclei (red arrows) within the Langerhans islets. Mean values ± standard deviation of TUNEL positive nuclei/µm² are presented in e. π p<0.05 vs. SSD; μ p<0.05 vs. ASD .......................................................... 124

Figure 5 – Representative light micrographs of pancreas sections immunostaining for NF-kB from physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d) groups, depicting Langerhans islets. The NF-kB positive nuclei are identified by the black arrows. Mean values ± standard deviation from all groups are depicted in e. π p<0.05 vs. SSD; μ p<0.05 vs. ASD; δ p<0.05 vs. AFD ................................................................. 125
LIST OF TABLES

REVIEW OF LITERATURE


Table 2. Associations between sedentarism and metabolic syndrome risk factors

STUDY I

Table 1. Macronutrients and diet characterization ............................................. 60
Table 2. Body Weight and Tibia Length characterization ................................. 65
Table 3. Morphometric features ................................................................. 67

STUDY II

Table 1. Diet characterization ........................................................................ 85
Table 2. Rank for Non-Alcoholic Fat Liver Disease activity score .................... 89
Table 3. Morphological characterization of BW, LW and rLW ....................... 90
Table 4. Occurrence of metabolic syndrome among groups ......................... 91
Table 5. NAFLD score components ............................................................. 92
Table 6. Macrophage phenotype and NF-kB activation assessment ................. 96
STUDY III

Table 1. Standard and high-fat diet composition ................................. 115

Table 2. Variables required for metabolic syndrome diagnosis ............... 120

Table 3. Absolute frequency of animals in each group with values of triglycerides, total cholesterol, blood pressure, and central obesity higher than the percentile 100 of physically active standard diet-fed (ASD) control group
........................................................................................................ 120

Table 4. Area under curve of glucose tolerance test and insulin sensitivity test
....................................................................................................................... 121

Table 5. Body weight and weight (absolute and relative) of pancreas and retroperitoneal adipose tissue ................................................................. 122
LIST OF ABBREVIATIONS

NCDs: non-communicable diseases

T2D: type 2 diabetes

CVDs: Cardiovascular Diseases

PIn: physical Inactivity

GLU: glucose

MS: metabolic syndrome

WHO: World Health Organization

ATP III: National Cholesterol Education Program Adult Treatment Panel III

IDF: International Diabetes Federation

NAFLD: nonalcoholic fatty liver disease

IR: insulin resistance

FFA: free fatty acids

BW: body weight
RESUMO

Esta tese é suportada por uma revisão narrativa da literatura, perspetivando fornecer uma visão geral dos principais aspetos etiológicos, fisiopatológicos e clínicos da síndrome metabólica, assim como por três artigos originais, utilizando um modelo animal, que objetivaram identificar a contribuição isolada e conjugada da inatividade física e de uma dieta hiperlipídica no desenvolvimento da síndrome metabólica. Para tal, ratos Wistar machos foram alimentados com dieta hiperlipídica e/ou tiveram a atividade física restrita ao espaço da gaiola por 21 semanas. Os resultados revelaram que a dieta hiperlipídica e a inatividade física induziram um característico fenótipo adulto, com ratos maiores e mais leves, possuindo órgãos mais leves e uma expressiva deposição de gordura retroperitoneal sem, contudo, ganho de peso. Os trabalhos empíricos também revelaram a predominância da dieta gordura sobre a inatividade física na indução de desarranjos metabólicos, uma vez que apenas os animais alimentados com dieta gorda desenvolveram síndrome metabólica, independentemente dos níveis de atividade física, com maladaptações hepáticas e do pâncreas endócrino evidenciando uma intensa resposta inflamatória, aumento do conteúdo local de colágeno e da apoptose celular em ambos os órgãos. Estes resultados permitem concluir que a dieta rica em gordura é um fator patogénico predominante na indução da síndrome metabólica e das maladaptações hepáticas e pancreáticas em um modelo animal, comparativamente à inatividade física. Ainda assim, comparativamente à dieta gorda, mais determinante na indução da intolerância à glicose, a inatividade física parece ser mais decisiva no comprometimento da sensibilidade à insulina. Níveis mais elevados de atividade física não parecem prevenir o desenvolvimento da síndrome metabólica e das alterações orgânicas associadas após 21 semanas de protocolo experimental, mas atenuam os transtornos metabólicos e estruturais induzidos pela dieta gorda.
ABSTRACT

This thesis is supported by a literature narrative review aiming to provide an overview of the metabolic syndrome main etiological, pathophysiological and clinical aspects, as well as three original articles using an animal model that aimed to identify the physical inactivity and the hyperlipidic diet isolated and conjugated contribution in the metabolic syndrome development. For this, male Wistar rats were fed with a high-fat diet and/or had the physical activity restricted to the cage space for 21 weeks. The results revealed that the high-fat diet and physical inactivity induced a singular adult phenotype, with larger and lighter rats, having lighter organs and an expressive retroperitoneal fat deposition without, however, gaining weight. The empirical studies also revealed the high-fat diet predominance over physical inactivity in inducing metabolic disorders, since only high-fat diet fed-animals developed metabolic syndrome, regardless of physical activity levels, with the hepatic and endocrine pancreas maladaptations evidencing an intense inflammatory response, increased local collagen content and cellular apoptosis in both organs. These results allow us to conclude that the high-fat diet is a predominant pathogenic factor in inducing the metabolic syndrome and the hepatic and pancreatic maladaptations in an animal model, compared to physical inactivity. Nevertheless, in comparison to the high-fat diet, which is more important in the glucose intolerance induction, physical inactivity seems to be more decisive in the impairment of insulin sensitivity. Higher levels of physical activity do not seem to prevent the metabolic syndrome development and related organic changes after 21 weeks of experimental protocol but attenuate the metabolic and structural disorders induced by the high-fat diet.
CHAPTER I

GENERAL INTRODUCTION AND AIMS
GENERAL INTRODUCTION

The early recognition of established homeostatic alterations can be a critical factor between the efficiency of preventive measures or the development of diseases, such as the non-communicable diseases (NCDs) like type 2 diabetes (T2D) and cardiovascular diseases (CVDs). NCDs have a complex origin directly linked to the interaction between genetic and environmental factors to which an individual is exposed during life [1]. However, despite the relative role of genetic factors for the susceptibility to disease development, the last decade has brought the environmental factors to the spotlight in NCDs development [2-4], mostly due to global changes in social and economic conditions [5].

Environmental factors can modulate metabolic traits predisposing NCDs development, especially those related to lifestyle features such as alcohol consumption, tobacco, unhealthy diets, and physical inactivity (PIn) [6, 7]. The combination of high caloric unbalanced diets with PIn usually at long-term results in weight gain and obesity, a clinical condition regularly related to metabolic and functional disturbances such as diminished insulin sensitivity, glucose intolerance, and hypertension [8-10]. Indeed, the link between obesity and the cluster of metabolic disorders belonging to the metabolic syndrome (MS) seems
to be irrefutable once the syndrome’s epidemic levels are concurrent with the massive rates of obesity around the world [11-13].

Costing around € 210 billion annually to the European community, the MS affects one-quarter of the global population and approximately 40% of Portuguese citizens, becoming a global public health problem that demands an urgent intervention [14-16]. The term metabolic syndrome was created in 1975 by Haller and Hanefeld [17] to illustrate the coexistence of metabolic disorders that significantly increases the individual's risk of developing CVD and T2DM [18]. It is assumed that the origin of the metabolic disturbances from MS is mostly based on the interaction between the genetic susceptibility and the environmental factors, mainly the factors belonging to the lifestyle as mentioned before [19]. Even so, identifying the clinical signs resulting from so many interactions, and uncovering the role of the environmental factors to the syndrome development is a constant challenge for clinical science. The predisposing factors diversity and the heterogeneity of its clinical manifestations along time result in a lack of clinical and academic consensus about the syndrome definition and diagnosis among worldwide researchers and health professionals. Indeed, even the leading guidelines from prestigious institutions such as the World Health Organization (WHO), National Cholesterol Education Program Adult Treatment Panel III (ATPIII) and the International Diabetes Federation (IDF) do not have a common
clinical definition for the syndrome’s diagnosis. Most of the controversies among specialists seem to be motivated by the MS components capacity to predict the development of CVD, DMT, and other related clinical conditions [20, 21].

Within the metabolic and clinical alterations of MS, liver and pancreas functionality assumes the spotlight since both organs are highly responsive to changes in the metabolic milieu and are both major effector organs of energy homeostasis and metabolic flexibility [22]. Usually called the syndrome’s hepatic manifestation, the non-alcoholic fatty liver disease (NAFLD) is the more prevalent cause of chronic liver illness in the Western world that regularly coexists with obesity, dyslipidemia and insulin resistance (IR) [23, 24]. Indeed, the NAFLD prevalence seems to simultaneously increase with the escalating incidence of MS [24, 25]. Likewise, the pancreas is also highly responsive to metabolic disturbances being a crucial thermostat of metabolic balance. It is assumed that the cluster of metabolic disorders from MS imposes a pancreas overload and requires a functional response, especially by the Langerhans islets, seeking to normalize disorders in energy metabolism generally related to the syndrome such as IR and glucose intolerance [26, 27]. Indeed, disorders in glucose and also insulin metabolism regularly emerge concomitantly with plasma lipoproteins abnormalities such as cholesterol and free fatty acid levels (FFA) [28], core traits of MS. The pancreas and liver long-term exposition to unfavorable environmental
stimuli, especially to unbalanced diets, and PIn since early stages of growth and development, can trigger phenotype responses that predispose to adulthood development of metabolic disturbances and NCDs [29, 30]. However, the adult phenotype of both organs, resulting from the long-term exposition to unbalanced diets and mostly to PIn during the growing and maturation phases, is still unknown.

In fact, the PIn role as an autonomous factor for pancreas and liver disturbances development has received less attention from the research community, even knowing that approximately 1.6 million of annual deaths are due to insufficient physical activity levels [31]. It is undeniable that the current literature does not provide a clear picture from the PIn impact on pancreas and liver disturbances related to MS or its components. While the methodological inconsistencies such as the adopted definitions for PIn classification and the predominance of subjective analyses of physical activity levels make the epidemiological evidence inconclusive, the evidence from animal models is mostly based on physical exercise programs that lead to substantial psychophysiological stress and do not mimic the animals’ pattern of physical activity in nature. Since the pancreas and liver are essential health indicators and critical thermostats of metabolic balance [32, 33], identifying structural and functional changes may be vital in predicting the early development of NCDs
related to the environmental factors that compose the lifestyle of an individual and a population. Especially the still unknown PIn impact, isolated or conjugated with an unbalanced diet, in the pancreas and liver phenotype related to MS.
AIMS

Considering all of the above, particularly the divergence and inconclusive evidence found in published research, the specific objectives of the research conducted for this thesis were:

1. To summarize the syndrome´s epidemiology, costs and the main etiological components related to environmental factors from lifestyle, specifically unhealthy diet patterns and physical inactivity;
2. To observe the environmental factors interference, namely fat diet and physical inactivity, since early ages in the adult morphological phenotype of Wistar rats;
3. To analyze the isolated and conjugated effect of physical inactivity and fat diet, isolated or conjugated with each other, on the development of MS and hepatic histologic profile in an animal model; and
4. To verify the isolated and conjugated impact of fat diet and physical inactivity in the endocrine pancreas phenotype related to overload in Wistar rats.

This thesis is structured according to the Scandinavian model and is divided into five chapters. The chapter I includes a brief introduction to the metabolic syndrome and environmental factors from lifestyle such as diet and physical inactivity and the thesis objectives. Chapter II consists of state of the art and includes a review from the syndrome´s epidemiology, costs and the main etiological traits from its relationship with unhealthy diet patterns and sedentary...
lifestyle. Next, **chapter III** is composed of three experimental studies related to the adult phenotype and the metabolic syndrome resulting from the animals’ long-term exposure to fat diet and physical inactivity, as well as the hepatic and pancreatic repercussions to both environmental factors. **Chapter IV** is dedicated to a general discussion of methods and the main results obtained in the original studies reported in the respective chapters. To conclude, **Chapter V** addresses the essential conclusions from the studies. Last, **chapter VI** presents the bibliography of the work that supports chapter I and IV.
CHAPTER II

STATE-OF-ART
REVIEW ARTICLE

Metabolic Syndrome: The Pathophysiologic Role of Diet and Sedentary Lifestyle

Antonio Bovolini a*, Juliana Garcia b, Maria Amparo Andrade c, José Alberto Duarte a*

a CIAFEL, Faculty of Sport, University of Porto; R. Dr. Plácido da Costa 91, 4200-450 Porto, Porto, Portugal.
b UCIBIO / REQUIMTE - Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto; R. Jorge de Viterbo Ferreira 228, 4050-313 Porto, Portugal.
c Federal University of Pernambuco; Av. Prof. Moraes Rego, 1235 - University City, Recife - Pernambuco, 50670-901, Brazil.

* Authors to whom correspondence should be addressed (Tel.: +351 22 04 25 200; Fax: +351 225 500 687; e-mail: jabovolini@hotmail.com and jarduarte@fade.up.pt).
Abstract

Metabolic syndrome is a cluster of cardiometabolic features with high prevalence rates in obese and overweight subjects with an elevated cost for public health systems worldwide. Despite the lack of consensus regarding the syndrome’s definition and diagnosis criteria, it is characterized by the coexistence of dyslipidemia, elevated blood pressure, prothrombotic and serum pro-inflammatory state, insulin resistance and higher plasma glucose levels, disorders indisputably linked to an increased risk of developing chronic conditions such as type 2 diabetes mellitus and cardiovascular diseases. The syndrome has a complex and multifaceted origin not fully understood; however, epidemiologic studies strongly suggest that the sedentarism and the unbalanced dietary pattern might play a fundamental role for the syndrome development. The purpose of this review is to provide an overview of the epidemiology data, analyzing the syndrome’s clinical and laboratorial components, as well as the potential role of life-style factors, particularly unhealthy diet patterns and sedentary lifestyle, on its pathophysiology and progression.

Keywords: Fat diet, Physical inactivity, Sedentarism, Syndrome X
Introduction

The metabolic syndrome (MetS) is a silent epidemic and represents a major public health problem worldwide. The syndrome encompasses a series of asymptomatic metabolic disorders such as the "traditional" disturbances like dyslipidemia, hypertension, insulin resistance (IR) as well as the "recent" recognized components like the pro-thrombotic and systemic pro-inflammatory state, making the syndrome one of the major risk factors for heart disease development [1]. Indeed, MetS confers a 5-fold increase in the risk of type 2 diabetes mellitus (T2D) establishment, 2-fold the risk of developing cardiovascular disease (CVD) over the next 5 to 10 years, 2- to 4-fold increased risk of stroke, a 3- to 4-fold increased risk of myocardial infarction, and 2-fold the risk of dying from such an event compared with those without the syndrome [2]. The MetS has a multifactorial and not entirely understood origin, however, the genetic predisposition associated with environmental factors linked with lifestyle have assumed the leading role in the syndrome establishment [2]. Lifestyle, particularly the globalization of dietary pattern and the increasing levels of sedentarism, might play a significant role in the development of metabolic syndrome components. However, the complex relationship between lifestyle factors and syndrome development remain unclear. Likewise, the methodological variability among experimental studies and the different findings reported by epidemiological studies also require a constant knowledge revision update. For these reasons, this review aims to provide the state-of-the-art concerning MetS, focusing on the relationship between MetS, diet, and sedentary lifestyle.

Metabolic Syndrome

MetS become the subject of several research groups worldwide and numerous published scientific works. At least, more than 48 thousand related studies were published in the last decade, according to the American search engine PubMed. The direct relationship between the volume of produced knowledge and the increasing MetS global prevalence is an undeniable argument to promote the discussion of this epidemic that no longer knows economic or socio-cultural barriers.
1. Definition

Despite being traditionally recognized as a constellation of CVD risk factors, the MetS have been subject to different definitions according to the components involved in its diagnosis. However, it is consensual among all available definitions that its diagnosis requires the association of, at least, two of the following components: central obesity, hypertension, dyslipidemia, and IR. Based on this, the International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) propose in 2009 the last attempt to unify the existing definitions [3], however without success. The most representative definitions and their main differences can be seen in Table 1.


<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Obesity</td>
<td>GT, IGT or T2DM and/or IR plus with two or more risk factors</td>
<td>three or more associated risk factors</td>
<td>central obesity (race- and gender-adjusted) plus any two of the following four parameters</td>
<td>three or more associated risk factors</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>≥140/90 mmHg</td>
<td>&gt;130/85 mmHg</td>
<td>≥130 mm Hg systolic or ≥85 mm Hg diastolic or on hypertension treatment</td>
<td>≥130/85 mmHg or on hypertension treatment</td>
</tr>
<tr>
<td>Lipid Profile</td>
<td>TGC ≥ 150 mg/dl and/or low HDL-C; &lt;35 mg/dl for men and &lt;39 mg/dl for women</td>
<td>≥150 mg/dl or chronic treatment for lipid abnormality and reduced HDL cholesterol: &lt; 40 mg/dl for males and</td>
<td>TGC ≥150 mg/dl or on TGC treatment HDL-C men &lt; 40 mmol/L women and &lt; 50 mmol/L</td>
<td>TGC ≥ 150 mg/dl or on TGC treatment HDL-C men &lt; 40 mmol/L women and &lt; 50 mmol/L or HDL treatment</td>
</tr>
</tbody>
</table>

WHR>0.9 for men and >0.85 for women and/or BMI>30 kg/m²
WC ≥102 cm in men and ≥88 cm in women
Increased WC (specific race- and gender- cutoffs) [7]
WC ≥102 cm in men and ≥88 cm in women

GT, IGT or T2DM = GTT, IGT or T2DM
2. Epidemiology and health costs

The IDF estimates that approximately a quarter of the world’s population is affected by the syndrome [7]. A similar prevalence was presented by the National Cholesterol Education Program (NCEP), estimating that the MetS affects more than 20% of the Western countries adult population [8]. However, factors such as sex, age, race, environment, and socio-cultural aspects can completely change the estimates. For example, according to Kolovou et al., the MetS prevalence could vary from 4% in healthy USA adolescents to 84% in Finnish and Swedish men diagnosed with T2D [9]. Likewise, the National Health and Nutrition Survey (NHANES) points to the incidence variation among individuals with different body mass indexes, about 5% in normal-weight individuals, 22% of overweight and impressive 60% among obese individuals [9, 10]. According to the NHANES, obese individuals have a 12-fold higher incidence than normal-weight individuals. The prevalence rates also reveal an expressive range of incidence among age groups and also a positive association between aging and MetS development, increasing from 10% in individuals between 20-29 years old to 45% in individuals aged between 60-69 years [11]. Likewise, sex and race/ethnicity can also influence the syndrome prevalence rates worldwide. Based on the criteria from the National Cholesterol Education Program’s Adult Treatment Panel III (NCEP/ATP III), Ervin et al. point out that approximately just 20% of males and 16% of females under 40 years of age reach the criteria for MetS diagnosis. In the population between 40–59 years of age, the prevalence rises to 41% in males and 37% in females, while in subjects
with 60 years of age and over it increases to 52% in males and 54% in females, indicating the increased prevalence of MetS with age, being slight higher in males [12]. The most recent data of Beltran-Sanchez et al. [13] examined the trends in MetS prevalence from 1999–2000 to 2009–2010 in U.S. adults. Considering the syndrome prevalence by ethnicity, just the Caucasian population (25.6% in 1999–2000 and 21.8% in 2009-2010) shown a diminished prevalence, whereas no differences were observed in African American (22.0% in 1999-2000 and 22.71% in 2009-2010) and Hispanic population (32.6% in 1999-2000 and 31.9% in 2009-2010) [13]. Independently the evaluated populations or the adopted stratification, data revealed a high prevalence of MetS.

Previously restricted to developed countries, the globalized and unbalanced way of life has become a global health problem and a primary target of public spending on health care. Indeed, it is expected that the MetS epidemic and its pathogenic cluster of risk factors have a high cost for public health systems worldwide. According to Sullivan et al. [14], MetS patients with an association of three or more components cost per year 60% more than subjects without MetS` disarrangements. Economically, the study indicates that MetS cost annually to the American public coffers around 5,477 dollars per patient, an expense 2,151 dollars higher than patients without the syndrome [14]. The costs become even more alarming when the association of components is higher than four MetS components, enhancing 24% more the expenses to the American pockets [14, 15]. The latest American Heart Association (AHA) study, which projects the prevalence and medical costs of CVD from 2017 through 2035, suggests that the expenditures with CVD resulting from MetS will cost to American funds an astounding 1 trillion of dollars [16]. In European Community, the European Heart Network (EHN) estimated that the CVD including nonhealth-care costs, such as productivity losses and public awareness, prices 210 billion euros annually to the 25 members countries, 111 billion euros only with health care expenses [17]. In the South Asia countries (India, Pakistan, Bangladesh, Sri Lanka, Nepal, Afghanistan, Maldives, and Bhutan) the diabetes-related care, costs in 2014 around 6.9 billion of American dollars according to estimates from the IDF [18]. In sub-Saharan Africa, just in 2011, the expenditures were $2.8 billion, and the IDF projections expect a 61% increase until 2030 [18].
The prevalence and the MetS shocking costs around the world show no signs of slowing down but instead, shows rising rates, making the syndrome management a global public health challenge. Considering the individual costs with the other syndrome components, the perspectives point to an unsustainable situation.

3. Etiology

The MetS etiology is not totally clear, but it seems that the syndrome results from the complex interaction between genetic and environmental factors [2]. However, epidemiological evidence from the last decades has pointed out to the environmental factors’ predominance over the genetic contribution in inducing the metabolic disorders belonging to the syndrome cluster. Among them, the environmental factors related to the lifestyle, mainly physical inactivity and unbalanced diet patterns, have been recognized as significant risk factors for developing MetS mostly mediated by obesity [19]. The interplay between genetic and environmental factor is complex and unclear.

- Genetic Factors

Despite the strong association between the syndrome and obesity, some lean individuals and ethnic groups have a high and paradoxical incidence/prevalence of MetS [13]. With an analogous paradigm, a significant number of obese individuals has been recognized as not expressing metabolic disorders [20], indicating that the syndrome etiology goes beyond the environmental factors influence. Data from 314 white adults from the southern part of Germany in which total body fat (visceral, and subcutaneous fat) was measured showed a “metabolically benign obesity,” not accompanied by insulin resistance and atherosclerosis development [20]. It is probable that the metabolic sensitivity to different environmental factors is under genetic control [21, 22]. The genetic predisposition may explain why lean subjects, such as southern Asian ethnic groups, or obese individuals “metabolically healthy” are, or are not, prone to develop MetS [23, 24]. The genetic predisposition to specific risk factors has been related to different types of genetic polymorphisms and linked to metabolic processes such as lipoprotein and insulin metabolism [25, 26]. Herein, Wang et al. [25] provided genetic evidence to support the hypothesis that the insulin receptor gene exon 8 variant is associated with the MetS pathogenesis in the Chinese population.
Moreover, MetS genetic predisposition was corroborated by a study of Chiefari et al., which demonstrates that the high-mobility group AT-hook-1 gene is a major susceptibility locus that confers a high risk for MetS development, predisposing to unfavorable anthropometric and metabolic traits [27]. Different studies also point out the heritability as a decisive factor for MetS development [28-30]. In an extensive review, van Dongen et al. [31] estimated the genetic influence on the components of the metabolic syndrome based on a series of studies. According to the authors, the genetic influence may vary from 24-90% for body mass index (BMI), 10-75% for fasting glycemia, and expressive 0.03 to 72% for high triglycerides levels, among other parameters. Indeed, recent evidence based on data from three large familial cohorts of whites and blacks has supported the heritability importance not only in MetS development but also in the syndrome severity [32].

However, the genetic predisposition also seems to be influenced by the environment changes at different development stages, as proposed by the thrifty phenotype hypothesis [33]. Firstly suggested by Neel in 1962 and posteriorly by Hales and Barker in 1992 [33, 34], the hypothesis proposed that restrictive environments or with an unstable energy intake on intrauterine phases favor the gene expression leading to an energy preservation phenotype. This genetic manifestation favorable in restrictive environments becomes disadvantageous when food conditions become better, supporting the development of pathologies related to energy unbalances due to its thrifty characteristics modulated in food restriction states.

- Environmental Factors Lifestyle-dependent

The genetic factors relative contribution to MetS origin and its components such as hypertension, IR, central obesity, and dyslipidemia, has been controversial [35]. Studies using an evolutionary perspective have demonstrated the role of gene-environment interactions in the development and increased incidence of MetS and associated illnesses such as T2D and CVD [36-38]. Indeed, experimental studies have been suggesting that prenatal factors can play a fundamental role in the origin of the metabolic syndrome, demonstrating that an adverse fetal and
even embryonic environment can induce both structural and functional abnormalities in the pancreatic islet cells leading to alterations in insulin metabolism and sensitivity [39, 40].

- **Diet**

  Dietary habits can play an essential preventive or pathogenic participation in weight gain, metabolic disorders, and consequently, MetS development [41]. Besides central obesity, the nutrients per se can also trigger inflammatory responses via biological molecules resulting from their metabolism [42]. Besides resulting in calories over-intake and weight gain, it is expected that the nutrients overconsumption, particularly carbohydrates and fat, potentiate the inflammatory process feeding a vicious cycle between diet, inflammation, and obesity which compromises insulin metabolism [43, 44]. Indeed, the excessive weight gain can promote the induction of inflammation in different tissues and stimulates the inflammatory cells migration such as macrophages and monocytes, mainly to the adipose tissue [45]. The carbohydrate consumption has been pointed out as a critical factor in inducing weight gain, obesity and also related diseases such as T2D and metabolic disorders, especially the overconsumption of simple and high processed sugars [46]. Substantial epidemiological evidence has been demonstrating the direct association between the diets intake with high concentrations of added-sugars with the development of unfavorable lipid profile [47, 48], IR [49, 50], non-alcoholic fatty liver disease [51, 52], T2DM [53, 54], visceral adiposity [50, 55], and MetS [50, 56] as well. However, the direct effects of sugar consumption in increasing risk factors for metabolic diseases in the absence of positive energy balance and overweight/obesity are still unknown. Likewise, current evidence indicate that both the quantity and quality of dietary fats can directly affect the serum lipid profile, being also directly related to obesity, T2D, and MetS [57]. Indeed, the high consumption of total fat has been strongly associated with fasting hyperinsulinemia [8,9], diminished insulin sensitivity [10], and also with the progression of glucose intolerance to T2DM in patients with diminished glucose tolerance [13]. The long-term exposure of metabolically active tissues and organs such as skeletal muscles, liver, and pancreas to serum lipids can lead to significant dysfunctions regularly related to inflammatory pathways activation and systemic IR [58]. However, little is known regarding associations of total fat and the type of fat intake with MetS and T2DM.
development. More than the excessive consumption of calories, growing evidence has demonstrated the relationship between the unbalanced dietary patterns and the development of T2D and other metabolic disorders [59, 60]. Among them, the ‘Western’ dietary pattern mainly characterized by red meat, refined grains, sweets, and sugar-sweetened beverages have gained prominence as a MetS risk factor [61-63]. Regularly associated with a sedentary lifestyle, the Western dietary pattern globalization has been implicated as a primary factor in the epidemic MetS in different world regions such as North America [64, 65], Europe [66], and the Middle East [66, 67]. Interestingly, studies in eastern populations such as Koreans and Japanese have not detected a significant association between the Western dietary pattern and the metabolic syndrome [68-70], countering robust epidemiological evidence that the dietary patterns “westernization” increases the risk of CVD. However, more longitudinal studies are needed to validate dietary patterns role in MetS development and to seek strategies to prevent chronic diseases development.

○ Sedentarism

Sedentarism (SED) or sedentary behaviors/activities (SB) does not have a consensual definition. It has usually been defined as “any behavior/activity characterized by an energy expenditure ≤ 1.5 MET, predominantly in a sitting position” [71]. However, it is important to note that SB does not imply the physical activity (PA) lack, but rather a predominance of SB along the day, such as working, driving or watching television [72]. For example, it is possible to achieve the daily recommendations of moderate/vigorous PA (MVPA) and be highly sedentary throughout the daily bases given the time sitting at work or resting. In fact, current trends related to the SB study have pointed out that SB and MVPA usually coexist, even in a daily active individual [73]. Nevertheless, Owen et al. [73] emphasize that SB are only significant if the time spent on SB contributes to the reduction of daily overall PA time. Even so, the presence of MVPA in the routine of an individual considered sedentary still confuses researchers around the world, being the subject of a letter that sought to clarify the different approaches related to the SB study [74]. In this letter, seeking to elucidate the terminology surrounding the sedentarism, the authors cite the two SB main used definitions/terminologies in scientific approaches. The authors describe
that studies carried out in the biological and public health areas consider sedentarism the predominance of all PA with an energy expenditure $\leq 1.5$ METS in a sitting or reclining position. While the academic works developed in physical exercise area usually are guided by the MVPA guidelines to classify sedentary individuals [74].

The SB adverse effects are undeniable in an individual's clinical profile. The SB became itself a premature death risk factor independently of sex and type of practiced PA by an individual [75]. In fact, it has been demonstrated that a sedentary lifestyle has a direct impact on life expectancy due to the mortality from all causes, but mainly due to CVD induced by harmful metabolic alterations commonly founded in diabetogenic and atherogenic profiles [76, 77]. Particularly the cardiometabolic risk markers such as BMI, waist circumference, blood pressure, TGC, HDL cholesterol, fasting, and postload plasmatic glucose, and fasting insulin as shown by Thorp et al. in the Australian Diabetes, Obesity, and Lifestyle (AusDiab) study [78]. Likewise, the lack of muscular contraction seems to be the SED chief damaging contributor to the cardiometabolic profile, inducing glucose metabolism, and triglyceride clearance disorders. Mechanisms such as muscular suppression of the enzyme lipoprotein lipase (LpL) expression, diminished insulin sensitivity, reduction of plasma glucose clearance due to abnormal GLUT4 translocation, and consequent decrease of insulin secretion by glucose stimulation has been found in SB individuals [77, 79]. Studies with populations with a higher SB prevalence, such as patients with different types of psychosis, have demonstrated the SB impact on life expectancy, about 15 years below the general population [80, 81]. Likewise, studies in the general population have also demonstrated the damaging relationship between a sedentary lifestyle and diminished life expectancy, especially studies on cardiometabolic health consequences of prolonged sitting time [82, 83]. Based on the results of the AusDiab study, an 8,800 adults follow-up for approximately seven years, Dunstan and colleagues [84] found that every hour spent on TV increased by 11% the risk of death from all causes and 18% death from CVD. Considering the significant number of the worldwide workers [85, 86] who spend up to 70% of the seated time [87], the 3.2 million premature deaths related to sedentary lifestyles estimated by the WHO is not surprising but rather expected [88]. Even so, the sedentarism rates are still growing worldwide.
The levels of sedentary behavior are not part of the MetS components; however, SB is highly related to the development of common comorbidities such as diabetes, CVD, cancer, increased risk of premature death and diminished life expectancy, independently of MetS presence [75-77, 82]. More than related, the SED has become by itself an independent risk factor for CVD and premature death due to its capacity to promote metabolic disorders [89-91]. The ability to generate metabolic imbalances passes mostly through its role in energy balance that leads to overweight/obesity [92]. A recent follow-up addressed the impact of SB on clinical parameters used to diagnosis the syndrome, such as BMI, waist circumference, blood pressure, lipid, and insulin profile, and the fasting and post-loading glycemic pattern regardless of PA levels [78]. Although the absence of direct data supporting a cause/effect relationship between them, SED should be qualified as a MetS risk factor, mostly due to the close relationship between SED and the components of the syndrome as shown in Table 2.

Table 2. Associations between sedentarism and Metabolic Syndrome risk factors.

<table>
<thead>
<tr>
<th>RISK FACTOR</th>
<th>PERIOD AND ASSESSMENT OF SEDENTARISM FOLLOW-UP</th>
<th>MAJOR OUTCOMES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8800</td>
<td>Interviewer questionnaires 6 years</td>
<td>Abnormal glucose metabolism risk.</td>
<td>Dunstan, D.W. et al. [84]</td>
</tr>
<tr>
<td>68497</td>
<td>Questionnaire 6 years</td>
<td>Directly related to diabetes risk.</td>
<td>Frank, B.Hu. et al. [93]</td>
</tr>
<tr>
<td>5964</td>
<td>Interview / prompt cards 2 years</td>
<td>Increased risk of incident diabetes mellitus.</td>
<td>Smith, L. et al. [94]</td>
</tr>
<tr>
<td>59052</td>
<td>postal questionnaires 10 years</td>
<td>Positive association between television watching and T2DM risk.</td>
<td>Krishnam, S. et al. [95]</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8800</td>
<td>Interviewer-administered questionnaires 6.6 years</td>
<td>Diminished life expectancy</td>
<td>Dunstan, D.W. et al. [84]</td>
</tr>
<tr>
<td>1693</td>
<td>Self-report questionnaire 3 years</td>
<td>Linearly associated with systolic and diastolic BP</td>
<td>Aadahl, M. et al. [96]</td>
</tr>
<tr>
<td>ID</td>
<td>Methodology</td>
<td>Findings</td>
<td>Reference</td>
</tr>
<tr>
<td>-----</td>
<td>----------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>11837</td>
<td>Biennial questionnaires 40 months</td>
<td>Interactive SB are associated developing hypertension independently of PA</td>
<td>Beunza, J.J. et al. [97]</td>
</tr>
<tr>
<td>8800</td>
<td>Interviewer-administered questionnaires 6.6 years</td>
<td>Diminished expectancy</td>
<td>Dunstan, D.W. et al. [84]</td>
</tr>
<tr>
<td>739</td>
<td>ActivPAL3™ physical activity monitor 1 year</td>
<td>Sitting Time were significantly and deleteriously associated with HDL and cholesterol triglycerides.</td>
<td>Bellettiere, J. et al. [98]</td>
</tr>
<tr>
<td>15515</td>
<td>Questionnaire 4 years</td>
<td>Significant positive associations between increasing television viewing, total cholesterol, LDL cholesterol and a significant inverse association between increasing television viewing and HDL cholesterol.</td>
<td>Jakes, R. W. et al. [99]</td>
</tr>
<tr>
<td>8800</td>
<td>Interviewer-administered questionnaires 6.6 years</td>
<td>Increased risk of all-cause and CVD mortality</td>
<td>Dunstan, D.W. et al. [84]</td>
</tr>
<tr>
<td>50277</td>
<td>Questionnaire 6 years</td>
<td>Significantly associated with obesity and T2D risk</td>
<td>Frank, B.Hu. et al. [93]</td>
</tr>
<tr>
<td>2449</td>
<td>ActivPAL physical activity monitor 3 years</td>
<td>PA and SB levels may partly explain the MetS presence in obese as well as non-obese individuals.</td>
<td>de Rooij, B.H. et al. [100]</td>
</tr>
<tr>
<td>15515</td>
<td>Questionnaire 4 years</td>
<td>Self-reported television viewing was positively associated with markers of obesity independently of PA</td>
<td>Jakes, R. W. et al. [99]</td>
</tr>
<tr>
<td>12409</td>
<td>Questionnaire 7 years</td>
<td>Occupational SB was positively associated with obesity risk</td>
<td>Nicholas, J.A. et al. [101]</td>
</tr>
</tbody>
</table>

**Dyslipidemia**

**Obesity**

**Proinflammatory / Prothrombotic State**
<table>
<thead>
<tr>
<th>Referential</th>
<th>Type</th>
<th>Years</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4512</td>
<td>Interview</td>
<td>4 years</td>
<td>Association between sitting time and CVD risk partly explained by the metabolic and inflammatory pathway's role</td>
<td>Stamatakis, E. et al. [102]</td>
</tr>
<tr>
<td>1543</td>
<td>Questionnaire</td>
<td>3 years</td>
<td>SB is associated with unfavorable levels of adiposity-associated inflammation.</td>
<td>Allison, M. A. et al. [103]</td>
</tr>
<tr>
<td><strong>Metabolic Syndrome</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8800</td>
<td>Interview-administered questionnaires</td>
<td>6.6 years</td>
<td>Diminished life expectancy</td>
<td>Dunstan, D.W. et al. [84]</td>
</tr>
<tr>
<td>18880</td>
<td>Computer-based questionnaire</td>
<td>4 years</td>
<td>Low PA levels and time watching TV were associated with MetS regardless of BMI and independently of each other.</td>
<td>Roos, V. et al. [104]</td>
</tr>
<tr>
<td>13017</td>
<td>Postal questionnaires</td>
<td></td>
<td>SB (viewing/computer use) are positively related to MetS.</td>
<td>Bertrais, S. et al. [105]</td>
</tr>
<tr>
<td>6400</td>
<td>Questionnaire</td>
<td>5 years</td>
<td>High levels of SB were associated with a greater prevalence of the MetS.</td>
<td>Gardiner, P.A. et al. [106]</td>
</tr>
<tr>
<td>20502</td>
<td>Questionnaire</td>
<td></td>
<td>Decrease of sedentary time reduces the prevalence of the MS and its abnormal components.</td>
<td>Xiao, J. et al. [107]</td>
</tr>
</tbody>
</table>

**ABBREVIATIONS:** BLOOD PRESSURE (BP); TYPE 2 DIABETES MELLITUS (T2DM); CARDIOVASCULAR DISEASE (CVD);

4. **Pathophysiology**

Several mechanisms have been proposed to unravel the MetS pathophysiology; however, as the term syndrome indicates, the MetS etiology is still not clear. Nonetheless, the visceral adiposity and IR seem to be the basis of MetS pathophysiology [108] that also include a proinflammatory and prothrombotic state, hypertension, and dyslipidemia as potential underlying mechanisms.
- **Visceral Adipose Tissue**

The contribution of adipose tissue to the syndrome’s pathophysiology is controversial due to the metabolic specificities related to its distribution. However, the central fat deposition, particularly the visceral adipose tissue (VAT), has been widely recognized as highly metabolic damaging and continuously associated with insulin resistance [109] and cardiovascular diseases [110]. In fact, the VAT intercession on MetS disorders seems to be well accepted once it is more closely associated with IR than general obesity [111, 112]. The VAT noxious ability has been extensively discussed but seems to be centered on hormonal [113] and immunological modulation [114, 115]. As well, unlike the subcutaneous fat tissue responsible for the systemic lipolysis residues, the visceral adiposity appears to be the primary source of free fatty acids (FFA), firstly to the liver through the portal circulation and then to the systemic circulation [116]. The VAT is also a vital source of cytokines that actively participate in the induction of metabolic disorders related to the MetS [117].

The VAT is associated with increased levels of plasma FFA, which in turns leads to ectopic lipid deposition and lipotoxicity [118]. The chronic exposure to high circulating levels of FFA plays a crucial role in overall cellular dysfunction of liver, pancreas, and skeletal muscle [119-121]. The liver has a central role in lipid metabolic pathways by uptaking, processing, and storing serum FFA. Usually, the liver response to high serum levels of FFA results in hepatic IR and local lipids accumulation, probably mediated by the dysregulation of hepatic glucose production and the increased hepatic de novo lipogenesis [122, 123]. In the pancreas, the high circulating levels of FFA have been related to beta (β) cells structural and functional damage as oxidative endoplasmic reticulum stress and cell apoptosis, affecting insulin synthesis and secretion [124]. Taking into account that VAT accumulation is, not exclusively, but typically associated with obesity or overweight, it is possible that the increased insulin demand in these states induces overload and results in β-cell dysfunction [125]. The muscular tissue exhibits a similar response to chronic and elevated levels of FFA than hepatic and β-cells, with ectopic muscle lipid accumulation and local IR [126]. Although the exact mechanisms are still unknown,
the intramyocellular lipid accumulation is the probable precursor of muscle IR due to increased fatty acid uptake related to chronic lipid overload and beta-oxidation impairment [127]. In addition, there is strong evidence that elevation of plasma FFA is leading to insulin resistance by inhibition of glucose transport and phosphorylation with a subsequent reduction in rates of glucose oxidation and muscle glycogen synthesis [128, 129].

The involvement of adipokines in MetS pathophysiology has been recurrently suggested by animals and humans studies [130, 131]. Elevated levels of adipokine have been consistently implicated in IR and obesity-related MetS, although the unknown mechanisms responsible for adipokines over secretion [132, 133]. A recent study showed strong evidence that the dysregulated production of “offensive” adipocytokines, such as plasminogen activator inhibitor (PAI-1), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), monocyte chemotactic protein–1 (MCP-1), and angiotensinogen, as well as the reduced levels of “defensive” adipocytokines, such as adiponectin and leptin, actively participate in the pathogenesis of obesity-associated MetS [134]. The study also demonstrated that elevated levels of FFA increased the oxidative stress in cultured adipocytes, leading to dysregulated production of adipocytokines, such as adiponectin, PAI–1, IL-6, and MCP–1. Indeed, several adipose tissue-derived cytokines secreted into the circulation can contribute to low-grade chronic systemic inflammation, atherogenic effects, and IR development [135-139].

Concomitantly, the visceral adipocyte hypertrophy and hyperplasia antagonistically exhibit leptin overexpression [140]. The hyperleptinemia presented by obese patients may indicate resistance to the anorexic effects of leptin considering its metabolic repercussion in appetite reduction and energy expenditure increase [141]. In fact, the leptin overexpression become by itself a significant contributor to the development of MetS. Adiponectin is also another key cytokine exclusively secreted by adipose tissue, modulating some fundamental metabolic processes such as glucose metabolism and fatty acids oxidation [142]. Low levels of this adipokine have been reported in animal and human MetS [143, 144]. Considered a “good” adipokine due to its metabolic properties, the reduction of the basal levels has been implicated in the syndrome pathophysiology. The adiponectin anti-inflammatory and insulin-sensitizing effects
are based on key mechanisms in energy metabolism such as AMP-protein kinase activation (AMPK), decreased expression of adhesion molecules and inhibition of TNF-α induced nuclear factor-kappa B (NF-κB) activation [145-147]. Given these beneficial metabolic effects, the modulation of adiponectin levels has itself become a therapeutic target not only in the MetS treatment [148] but also in other important pathologies such as cancer [149].

TNF-α was the first proinflammatory cytokine related to the pathogenesis of IR and T2D in humans [150]. The TNFα deleterious effects have been widely studied in recent decades, especially for its active involvement in the IR induction through the inhibition of the insulin receptor substrate-1 (IRS-1) signaling pathway [151]. As well, the TNF-α also trigger the transcription of both NF-κB and the activator protein 1 (AP-1), increasing the proinflammatory cytokines production and exacerbating inflammation [152]. Combined with TNF-α, the IL-6 occupies a central place in the syndrome pathophysiology mediating mainly the inflammatory process through the stimulation of NF-κB [152]. IL-6 is also responsible for triggering the cytokines proinflammatory cascade, inducing the hepatic production of C-reactive protein (CRP) via portal vein [153]. This interleukin also plays significant roles in the appetite control and energy consumption by specific receptors expressed in the hypothalamic region of the brain [154], and in energy metabolism, such as abnormal insulin function, impaired glucose metabolism and consequently IR [155, 156]. In fact, its harmful effects have a strong basis in MetS pathophysiology. However, the IL-6 role in health and disease has been ambiguous, and evidence has suggested that IL-6 can also play beneficial roles in both glucose homeostasis and energy balance [157, 158]. Moreover, despite the long and well-established damaging potential list, the new and counter-productive evidence brings IL-6 back to the limelight.

The PAI-1 production by adipocytes is highly responsive to pro-inflammatory cytokines and ROS, especially cytokines related to VAT and hepatic ectopic fat such as TNF-α, transforming growth factor beta (TGF-β), and insulin [159]. Strongly connected to MetS pathophysiology, PAI-1 has become an important biomarker to evaluate the syndrome severity due to its association with IR, non-alcoholic fatty liver disease, a proinflammatory and prothrombotic state [160, 161]. The significant contribution of PAI-1 to the MetS cluster may link to the establishment of a
prothrombotic state as found in patients with elevated circulating levels of this cytokine. Acting together with the fibrinogen, the PAI-1 contributes to the formation of atherothrombotic events. The high reactivity of PAI-1 and fibrinogen to proinflammatory cytokines, such as CRP and IL-6, also suggests the interrelationship between the proinflammatory and prothrombotic states in MetS pathophysiology [162, 163].

- **Insulin Resistance**

Several mechanisms have been proposed to elucidate the occurrence of IR in MetS. Among them, the fatty acids mediation, especially saturated fatty acids, play a critical role in the insulin signaling disturbances mainly through the activation of essential kinases in insulin signaling such as the protein kinase C (PKC) family, the inhibitor of nuclear factor kappa-B kinase-β (IKK-β), and the c-Jun N-terminal kinases (JNK) [164]. The activation of such cytokines induces disturbances in the signaling cascade after insulin receptor substrate-1 (IRS-1) phosphorylation, inhibiting the IRS-1 action [165]. Another suggested mechanism is that the abundant supply of fatty acids might increase the intracellular competition with glucose for substrate oxidation, leading to inhibition of key enzymes in energy homeostases such as phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH) [166]. The increased fatty acids delivery can also overcome the cell's capacity to oxidize fat and convert diacylglycerols (DAG) into triacylglycerols, resulting in intracellular accumulation of DAG, fatty acyl-coenzyme A (fatty acyl-CoA), and ceramides [167]. The intracellular accumulation of fatty acids metabolites like those previously mentioned has also been pointed out as a significant factor in disturbing the insulin signaling. Elevated intracellular levels of these metabolites usually result in serine/threonine phosphorylation IRS-1 and -2, a diminishing activation of phosphatidylinositol-3-OH kinase (PI3K), and an increased intracellular concentration of glucose declining the glucose uptake [167].

Moreover, upon reaching insulin-target tissues, mainly the skeletal muscle and liver, the excessive fatty acids availability usually induce IR modulating the downstream signaling cascade in these tissues [168]. Indeed, the fatty acids interference on insulin intracellular signaling pathways has also been demonstrating in skeletal muscle [169]. Evidence from humans an animal models of IR and T2D identified defects in the mitochondrial oxidative phosphorylation and also in
endoplasmic reticulum-related to the accumulation of triglycerides and lipid metabolites as determinant factors in the induction of muscular IR [170, 171]. High levels of fatty acids, particularly the saturated fatty acids, have been linked to decreases in IRS-1 tyrosine phosphorylation, diminished IRS-1 and -2-associated PI3-kinase activity, and a decreased phosphorylation and activity of protein kinase B (Akt) [172]. In the liver, the accumulation of fat metabolites, particularly DAG, has also been implicated in the hepatic IR due to the activation of the PKC family [173]. Indeed, the hepatic IR is mainly induced by defects in phosphorylation of insulin-stimulated receptors (IRS-1 and -2) related to activation of protein kinase C-ε (PKCε) but also the activation of c-Jun N-terminal kinase 1 (JNK1) [123]. Additionally, the excessive supply of fatty acids can also trigger the activation of inflammatory toll-like receptors (TLR) signaling, increasing the de novo ceramide synthesis and accumulation, and eventually inducing liver IR by Akt inhibition [173]. The Akt inhibition feeds a vicious hepatic circle that increases the transcription of key enzymes from the gluconeogenesis process [174].

- **Proinflammatory and Prothrombotic State**

The inflammation seems to be a pathophysiological link between obesity and metabolic disorders through the activation of the immune system [175]. The increased amount of immune cells as macrophages and acute phase proteins, such as a CRP, modulates the VAT cytokines production towards to a pro-inflammatory profile and a silent low-grade inflammatory state establishment [176]. The low-grade inflammatory state is regularly related to disorders of energy metabolism, especially insulin signaling disturbances in target tissues [114, 175]. Indeed, the loss of inflammatory control has been increasingly linked to muscular IR, failing insulin production and secretion related to β-cells loss, hepatic damage such as cirrhosis, sepsis, and even acute liver failure due to regenerative capacity suppression [177-179].

Synergistically with the pro-inflammatory state, the prothrombotic environment has also been considered crucial to the development of CVD [180]. Regulated by microRNAs but mainly by prothrombotic adipokines such as MCP-1, leptin, PAI-1, resistin, and visfatin, the establishment of a favorable environment for atherosclerotic incidents seems to be highly responsive to a
proinflammatory state [181]. In their review, Blokhin et al. [182] suggest that the etiology of the thrombotic event goes through chronic inflammation and fibrinolytic impairment, which can trigger the endothelial dysfunction, plaque rupture with tissue factor exposure, platelet activation, and late clot fragmentation.

- **Hypertension**

  One of the most important constituents of the syndrome, the hypertension is a transitory or sustained elevation of systemic arterial blood pressure (BP), which can induce cardiovascular damage and other adverse effects [183]. It is defined as systolic BP values ≥140 mmHg, and diastolic BP values ≥90 mmHg [184]. Evidence indicates that hypertension affects approximately 85% of MetS patients, being 50% of these patients insulin-resistant [185], with obesity playing a vital role in hypertension-related MetS [186].

  Some possible factors such as visceral obesity, IR, oxidative stress, endothelial dysfunction, renin-angiotensin system activation, increased inflammatory mediators, and obstructive apnea can trigger MetS' hypertension [187]. Such factors may produce sympathetic overactivity, peripheral vasoconstriction, increased intravascular fluid, and decreased vasodilatation, contributing to hypertension development in the MetS [188]. Although more research is still needed, ideally using large patient populations to understand better the underlying pathophysiology mechanisms behind the syndrome, there is a general agreement that VAT and IR are the core of most cases of hypertension in MetS [189]. The resistance to insulin-mediated glucose disposal can also determine compensatory hyperinsulinemia to maintain glucose homeostasis. This adaptive mechanism ultimately may promote hypertension and several atherogenic processes through mechanisms still to be discovered [185]. Visceral obesity also produces many adipokines that induce baroreflex dysfunction, an essential part of the hypertension etiology [188]. On the other hand, insulin also has systemic actions which could affect kidneys and sympathetic nervous system creating more clinical conditions for hypertension development [190]. IR can also increase sodium absorption and sympathetic activity that promote blood pressure elevation by the increase of circulating noradrenaline concentration in response to hyperinsulinemia [190]. Furthermore, oxidative stress and endothelial dysfunction have also been
associated with the development of hypertension in MetS patients [188], in a highly damaging vicious circle.

- **Dyslipidemia and lipid profile**

The serum lipoproteins pattern is an important predictor for coronary artery disease, especially the combination of high levels of triglycerides (TGC) and low-density lipoprotein cholesterol (LDL) allied to low levels of high-density lipoprotein cholesterol (HDL) [191]. Directly related to atherogenic events, dyslipidemia is one of the pillars of the MetS. Atherogenic lesions in MetS are mostly mediated by abnormal insulin metabolism and a series of consequent metabolic disorders [192]. Namely, the disorders in insulin signaling are generally responsible for increased lipolysis and circulating FFA, followed by a progressive increase in hepatic TG production related to increased FFA availability. In a positive feedback cascade, both insulin signaling disruption and increased FFA circulating levels stimulate the production of VLDL through the modulation of apolipoprotein B (apoB) and lipoprotein lipase (LpL) expression [193]. The IR states can directly act on the hepatic apoproteins synthesis, on LpL reduction, and the cholesterol transport protein (CETP) function [194]. In fact, increased IR results in a deficiency of lipoprotein lipase, an enzyme responsible for low clearance of fasting and postprandial triglyceride-rich lipoproteins (TRLs), and decreased production of HDL [195].

**5. Animal models of MetS**

Replicating the human MetS conditions is a challenge for scientists around the world given its complex pathophysiology. Since the human syndrome is undeniably linked to overweight and obesity, animal models of obesity have gained prominence, especially rodents [196]. Since then, models of MetS induced by dietary manipulation have been commonly used since they mimic better the clinical features of the human syndrome when compared to pharmacological and genetic models that contradict its polygenic origins [197]. Between them, Panchal and Brown [198] emphasize diets with high carbohydrate and fat content as the most successful eating pattern for MetS induction. Rodents regularly exposed to diets enriched with carbohydrate and fat present clinical signs such as overweight and obesity, abdominal and hepatic fat deposition, insulin
resistance, glucose intolerance, hypertension and endothelial dysfunction among other metabolic derangements from the human syndrome [199-203]. This nutritional combination replicates the human dietary patterns called "Western or cafeteria diet" being probably the reason why it has become one of the most used MetS experimental models. Even so, there are a variety of nutritional patterns also used to reproduce the syndrome. Wong et al. [204] recently reviewed other usual nutritional approaches highlighting too the solo use of high levels of fructose, sucrose, and fat to induce obesity-related MetS. Nevertheless, despite the dietary induction successful models, faster methods such as genetic manipulation and chemical/pharmacological induction have also become common laboratory practices in MetS study. In fact, the use of genetically obese and diabetic rodents via metabolic disturbances of leptin and its receptors, and models of glucocorticoid- and antipsychotic-induced MetS are also established research models [198, 204, 205]. However, regardless of the temporal advantage over dietary models and its usefulness on MetS mechanisms study, those models present a monogenic origin that contradicts one of the leading human syndrome features, its complex pathophysiology [197].

Although the different animal models of MetS have particular advantages and disadvantages, the pathophysiological similarity with the human syndrome, the reproducibility, and also the ethical boundaries have made the animal models a reliable tool for human syndrome replication and research. In fact, mammal models, including rodents, are essential in the MetS components study such as the body fat distribution and dysfunction, the glucose and insulin metabolism, and the immune system among key aspects from MetS as recently reviewed by Kleinert et al. [205]. Even so, pursuing the improvement and development of models capable of mimicking the human syndrome and its pathological mechanisms are fundamental in the prophylactic and therapeutic tools research.

6. MetS management

As discussed before, the MetS etiology is intrinsically linked to genetic and lifestyle-related environmental factors [2]. However, the lifestyle-related factors have been widely recognized as
dominant risk factors in inducing MetS mediated by obesity, mostly resulting from the positive energy balance and excessive weight gain [19]. Based on this, the lifestyle intervention has become the first therapeutic line against MetS. Frequently linked to states of overweight and obesity, the lifestyle changes mostly aim the body weight loss and anthropometric features in MetS treatment [206]. To achieve and potentiate weight loss, the caloric intake reduced, and the increase in energy expenditure are the most recommended non-pharmaceutical approaches. Indeed, even small losses in BW (5–10%) has been demonstrated to be sufficient to counteract MetS and its components individually [207, 208]. Also designed as a therapeutic lifestyle change by the NCEP ATP III, alterations in lifestyle have been continuously recommended to treat and reduce the syndrome epidemic feature [206]. Given its protagonism in MetS epidemic, the management of diet (mainly caloric restriction and foods quality improvement) and physical activity levels have become the main therapeutic tools in lifestyle changes related to MetS treatment. It is important to emphasize that MetS treatment also include the control of alcohol consumption, body weight and also the body fat distribution (waist circumference <94 cm in men, <80 cm in women), and smoking cessation [209].

In contrast with the western diet noxious nutritional composition, diets containing a low concentration of saturated fat and simple (and refined) carbohydrate have been massively recommended to reduce the caloric consumption and also to improve the metabolic profile [210]. Likewise, moderate consumption of red meat and alcohol has also been part of the dietary recommendations for MetS treatment and prevention [211]. Indeed, epidemiologic and clinical trials have been demonstrating the effect of adopting a balanced diet in MetS treatment but also its beneficial impact on MetS components individually, even being a high-fat diet pattern [210]. However, it is essential to highlight that the fat from high-fat diets patterns must be predominantly unsaturated and from vegetable origin [212]. The effects include the lowering of serum cholesterol and TGs levels, increased HDL cholesterol, improved glycemic control, decreased blood pressure, reduced central adiposity, and antioxidant and anti-inflammatory effects [213]. These effects are probably associated with the low glycemic apport and with nutrient density
presented by this type of food pattern, also known as the Mediterranean diet (MedD), which contains well-known bioactive food elements, mainly unsaturated fatty acids, complex carbohydrate, fiber, and vegetable protein [213]. Indeed, some studies have pointed out that the quality of diet components, namely the fats and carbohydrates, is more important than the amount of these macronutrients [213, 214]. Besides the MedD, other "healthy" diet patterns such as the Dietary Approaches to Stop Hypertension (DASH) diet, or dietary patterns assessed by the Alternative Healthy Eating Index, for example, has been inversely associated with the risk for MetS and T2DM development [214]. Dietary patterns that also contain high concentrations of the nutrients cited before, similar to MedD pattern (mostly plant-based) [213, 214].

Physical activity has a beneficial impact on most MetS components, preventing T2D and CVD development [215]. Regularly associated with diet control, higher levels of daily PA levels increase the energy expenditure impacting the caloric balance favorably [215]. Epidemiological data have continuously suggested that regular PA is an effective tool to prevents unhealthy weight gain and obesity. Indeed, evidence showed that the amount of PA reducing fat mass is dose-dependent, regardless of body weight changes [216, 217]. The PA effects on MetS management seems to be mostly based on its impact on body composition, mainly the reduction of visceral fat, with or without caloric restriction in overweight and obese individuals [218-220]. Decreases in BW, mainly the decreases in VAT, modulate the MetS components positively [221, 222]. Vast evidence has shown the beneficial effects of regular PA on insulin sensitivity, serum lipid profile, blood pressure, endothelial function, and also on the low-grade inflammation characteristic of the MetS [223-226].

7. Conclusion

The MetS have been emphasized as a significant socioeconomic problem all over the world, regardless of social class or ethnicity. However, although the organizations bring forward high prevalence rates, the distinct definitions and diagnostic criteria make impossible an accurate evaluation of the syndrome's prevalence and incidence. Considering the significant public
expenditures in the syndrome’s treatment or its components individually, a universal definition becomes imperative, allowing an early and more effective diagnosis. Thus, besides the diagnosis standardization, the syndrome early prevention is also imperative since the MetS’ components treatment, as well as related chronic diseases such as T2D, has a massive economic impact in public health care worldwide. An early diagnosis with an effective intervention will avoid the establishment and severity of MetS components and related chronic diseases, mainly T2D. The sedentary behavior and unbalanced dietary patterns are critical pathological factors in inducing metabolic disorders belonging to the MetS cluster. Given sedentary behavior and unhealthy diets as main environmental factors related to lifestyle in MetS origin, the lifestyle interventions are most effective therapeutic approaches to the syndrome and underlying chronic diseases.
8. Bibliography


Tumova, J., M. Andel, and J. Trnka, Excess of free fatty acids as a cause of metabolic dysfunction in skeletal muscle. Physiological research, 2016. 65(2).


Li, T., et al., High sucrose-fat diet and isosorbide mononitrate increase insulin resistance, nitric oxide production and myocardial apoptosis in a hypertensive rat model. Molecular medicine reports, 2018.


CHAPTER III

ORIGINAL STUDIES
STUDY 1

Antonio Bovolini, Maria Amparo Andrade, José Alberto Duarte (2019). *Influence of diet and physical activity levels, applied from the early stages of growth and development, on the adult phenotype of Wistar rats.*

Submitted to *Evolution and Development.*
Influence of diet and physical activity levels, applied from the early stages of growth and development, on the adult phenotype of Wistar rats.

Antonio Bovolini a*, Maria Amparo Andrade b, José Alberto Duarte a*  

a CIAFEL - Laboratory of Biochemistry and Experimental Morphology, Sports Faculty, University of Porto; R. Dr. Plácido da Costa 91, 4200-450 Porto, Porto, Portugal.  
b Federal University of Pernambuco; Av. Prof. Moraes Rego, 1235 - University City, Recife - Pernambuco, 50670-901, Brazil.  

* Authors to whom correspondence should be addressed (Tel.: +351 22 04 25 200; Fax: +351 225 500 687; e-mail: 201108996@fade.up.pt and jarduarte@fade.up.pt)
Abstract

Aim: This study aimed to analyze the fat diet and physical inactivity (Pln) influence on rodents’ adult phenotype. Methods: 40 Wistar rats were divided into physically active and inactive groups, standard or fat diet-fed (respectively ASD, AFD, AHFD, and SHFD). Active groups had free access to running wheel while the sedentary groups were restricted to cage space. Food consumption (FC), caloric intake (CI) and body weight (BW) were recorded weekly. Body mass variation (pBMV) was evaluated. After ending the protocol, the animals were weighed and euthanized. Liver, pancreas, retroperitoneal adipose tissue (RAT), heart, gastrocnemius and soleus muscle, and brain were excised and weighed and tibia length (TL) measured. Results: HFD-fed groups presented a significantly lower FC and CI whereas SD-fed groups a significantly higher carbohydrate and protein intake. HFD-fed groups exhibited a lower relative BW, pBMV and larger absolute TL values, being the normalized TL larger in active animals. HFD-fed animals also revealed a significant increase in RAT, reduced absolute and relative weight of gastrocnemius and pancreas, and a significant decrease in relative liver weight. Beside lighter than SD-fed animals, the HFD-fed animals had longer tibiae when normalized by BW. Conclusion: Diet and Pln modulation conditionate the animal’s adult phenotype.

Keywords: fat diet, sedentary behavior, rodents growing
1. Introduction

The environment plays a crucial role in growth and development, being able to guide and even determine life-history trajectories. Environmental factors such as temperature, diet, and lifestyle can define more than the life quality of an individual, but also biological features such as aging rating and life expectancy through a complex interaction between environmental and genetic factors (Barnes & Ozanne, 2011; Gilbert, 2001). Different environmental stimuli can actively induce variations in phenotypes shaping individuals regarding their physiological, morphological and metabolism specificities (West-Eberhard, 2003). In fact, the environment influence seems to be not just a coadjuvant factor in biological processes, but instead one of the protagonists of growth, maturation and development processes (Costantini & Monni, 2008; Monaghan, 2007).

Regularly used as equals, the terms growth, maturation, and development refer to different and specific processes (R. M. Malina, Bouchard, & Bar-Or, 2004). While development is largely a social and biological behavior expression linked to cultural specificities, growth and maturation refer to biological processes (R. Malina, 2008). Growth specifically denotes changes in bodily tissues and organs, requiring gains in weight, height, and alterations in body proportions, and is usually defined as a measurable and quantitative biological characterization (R. M. Malina et al., 2004). Instead, maturation is a process that, despite progressing to, is different from biological maturity, i.e., maturation is a process while maturity is a state (R. M. Malina, 2014).

Among the many influencing factors, diet and physical activity are possible determinants of the growth, maturation, and development processes (English & Uller, 2016; R. M. Malina et al., 2004; Taborsky, 2006). Meta-analyses, literature reviews, and experimental approaches have been demonstrating the early nutrition effects on growth and development (English & Uller, 2016; Nakagawa, Lagisz, Hector, & Spencer, 2012). Indeed, dietary modulation in experimental models induced immune system abnormal responses and also morphological and behavioral effects in adult animals when exposed to certain nutrition patterns since early development stages (Ohlsson & Smith, 2001; Royle, Lindström, & Metcalfe, 2005). As well, evidence suggests that physical
activity or its lack also affect the growth and development processes in a reciprocal relationship (R. M. Malina, 2014). This double influence path reflecting the complex interaction between neuromuscular maturation, growth, and environmental factors mainly experienced in the earliest life stages (Gilbert, 2001; R. M. Malina, 2014), requires adaptive responses to the demands imposed by environmental conditions and seems to be critical in the adult phenotype definition (Mulligan, 2016). However, the prolonged exposure to unfavorable stimuli provided by environment during growth and development stages, may trigger phenotype responses predisposing to adulthood pathologies and diseases. These maladaptive probabilities induced by environmental factors, such as diet and sedentarism, is also known as developmental origins of health and disease hypothesis (DOHaD) (Heindel, Skalla, Joubert, Dilworth, & Gray).

The analysis of responsive phenotypes to specific environmental factors has become an essential research tool in the identification of clinical signs and links between nutrition, physical activity/sedentarism, epigenetic processes, and the late disease development. Therefore, the manipulation of factors such as diet and physical activity became the center of numerous scientific works seeking efficient prophylactic and therapeutic strategies against non-communicable diseases (Koivusalo et al., 2016; Smith, Crippa, Woodcock, & Brage, 2016). However, most of them focus on exercise and caloric restriction in fetal or early-life developmental stages (Branquinho, Crepaldi, de Godoi, Pedrosa, & Baroni, 2018; Mangwiro et al., 2018; Nowacka-Woszuk, Madeja, & Chmurzynska, 2017; Włodek et al., 2017) and, despite the increasing number of studies in the field, the long-term effects of unbalanced non-restrictive diets are still unknown. Furthermore, in animal models it is known that imposed physical exercise programs lead to substantial psychological stress with further maladaptations, since these imposed protocols do not respect the rodents’ pattern of physical activity in their native environment (Moraska, Deak, Spencer, Roth, & Fleshner, 2000).

Moreover, given the active nature of rodents, it does not make sense to classify as controls the animals in which activity is restricted to their cage space, in opposition to those submitted to a training program, usually classified as experimental. Seeking to avoid all these methodological
misconceptions, this study aimed to observe the environmental factors interference since early ages, namely fat diet and physical inactivity, on the adult morphologic phenotype of Wistar rats. In our study design, animals with free access to a running wheel for voluntary running composed the control group, while sedentary animals, which physical activity was restricted to their cage space, constitute the experimental group.

2. Materials and Methods

2.1. Animals and diet

This study had the approval of the local ethical committee and animals’ housing and experimental treatment were in accordance with the guidelines defined by the European Council Directive (2010/63/EU) transposed into Portuguese law (Decreto-Lei n.º 113/2013, de 7 de Agosto).

Forty Wistar male rats (4 weeks old, 183.55 ± 3.48 g) provided by Charles River Laboratories (Barcelona, Spain) were housed individually in a room with controlled temperature (21-22°C) and humidity (50-60%) and inverted 12:12-h light/dark cycle. After 1-week of acclimatization fed a standard diet, animals were randomly divided into four groups: sedentary animals fed with high-fat diet (SHFD, n = 10), active animals fed with high-fat diet (AHFD, n = 10), sedentary animals fed with standard diet (SSD, n = 10) and active animals fed with standard diet (ASD, n = 10). Throughout 21-week experimental protocol, the animals were fed a standard diet (A04) or high-fat diet (diet purified HF 231), both from Scientific Animal Food & Engineering (SAFE, France). All groups had access to food and water ad libitum, and dietary consumption and body weight were recorded weekly in a precision balance (Kern 870, Balingen, Germany) for caloric and macronutrients consumption assessment. All data regarding energy density, diet composition, and the respective energy contribution from the macronutrients were provided by diet manufacturer (SAFE, France) and described in Table 1.
Table 1. Macronutrients and diet characterization.

<table>
<thead>
<tr>
<th></th>
<th>SD</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macronutrients Composition</td>
<td>Energy Contribution Kcal</td>
</tr>
<tr>
<td>Fat</td>
<td>3.1%</td>
<td>8.4%</td>
</tr>
<tr>
<td>Protein</td>
<td>16.1%</td>
<td>19.3%</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>60.4%</td>
<td>72.4%</td>
</tr>
</tbody>
</table>

SD, Standard Diet (3.339,00 Kcal/Kg); HFD, High-Fat Diet (5.335,80 Kcal/Kg). Data regarding energy contribution and diet composition were provided by SAFE (France).

2.2. Voluntary physical activity and imposed physical inactivity.

After the acclimatization period of one week, animals from the voluntary physical activity groups were individually housed in cages equipped with an activity wheel and distance counter. The running distance was recorded weekly. Animals belonging to the sedentary group were individually housed in cages without activity wheel and their physical activity was restricted to the cage space. Both cages had an identical floor area of approximately 800 cm² (Tecniplast, Buguggiate, Italy).

2.3. Sample collection and morphometric measurements

After the experimental protocol, animals were weighed, anesthetized with an intraperitoneal injection of Ketamine (90 mg/kg, Merial, France) and Xylazine (10 mg/kg, Bayer, German) and further euthanized by exsanguination. Liver, pancreas, retroperitoneal adipose tissue, heart, skeletal muscles (gastrocnemius and soleus), and brain were excised, rinsed in NaCl 0.9% solution and weighed with a precision balance (Kern 870, Balingen, Germany) for mass assessment. The right tibia was removed and measured for growth standardization. The percentage of body mass variation (pBMV) in experimental groups was performed comparing to their respective control groups using the adapted formula of Guarnieri et al. (Guarnieri et al., 2010):
% Body mass variation = \left( \frac{(ibm - fbm + cgbmg)}{(ibm + cgbmg)} \right) * 100

(ibm: initial body mass; fbm: final body mass; cgbmg: control group body mass gained)

Positive variation values represent body mass gain, and negative variation values denote decrease or cost in body mass variation when compared with the control group.

2.4. Statistical analysis

The Kolmogorov-Smirnov test was used to assess the normality of the data. For data without normal distribution, it was used the Kruskal-Wallis test followed by Dunn's test. Data with normal distribution were treated with One-Way ANOVA, followed by Tukey’s post hoc test. For the distance traveled and body weight analysis during the protocol was used the ANOVA repeated measurements. Data with normal distribution were presented as the mean±SD (standard deviation), and differences were considered significant when P<0.05. The software GraphPad Prism 7 (GraphPad Software Inc, La Jolla, CA, USA) was used for statistical analysis.

3. Results

3.1. Physical activity levels of animals physically active

Data regarding running distance is depicted in Figure 1. Interestingly, except the first and second weeks, the physically active animals fed with a fat diet demonstrated a significantly higher level of voluntary physical activity during the entire protocol in comparison to the physically active animals fed with a standard diet (p<0.0001).
3.2. Diet modulation and physical inactivity effects on food consumption and caloric intake.

The imposed physical inactivity and diet manipulation presented interesting results regarding food and caloric intake. The groups fed with SD showed much higher food consumption than animals HFD-fed (P <0.05 vs. SHFD and AHFD) (Figure 2). The results point a difference in the average dietary consumption by SD-fed groups, four times higher than HFD-fed groups with conditioning repercussions on caloric and macronutrient consumption. Conditioned by dietary intake outcomes, the caloric intake was substantially lower SHFD group (P <0.05 vs. SSD and ASD), even with the difference in energy density between both diets. On average, the SHFD animals presented an impressive caloric intake 41% lower compared to SD-fed groups average, even despite the fat diet presented an energy density 59% higher than the standard diet. Emphasis on the lowest and most unexpected caloric intake presented by the sedentary group administered with the HFD, 403.6±89.43 Kcal per week (P <0.05 vs. SSD and ASD). However, although the results demonstrate a higher caloric intake trend by active animals, the sedentarism seems to have not influenced both parameters between groups fed the same diet, any statistically significant differences between them were not found.
Figure 2. Mean values (±Standard deviation) of total food consumption and total caloric intake are depicted in A and B, respectively, in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups. The mean values variation for each group on food consumption and caloric intake along the experimental protocol are observed in C and D, respectively. (* p<0.05 vs. SSD; *** p<0.001 vs. SSD; ††† p<0.001 vs. ASD; ‡‡‡ p<0.0001 vs. AHFD).

Differences in diets composition were also expressed as relevant results regarding macronutrients consumption and caloric contribution. With low-carbohydrate and high-fat content, the HFD-fed group's primary energy source was the fat content, approximately 13 times higher than SD. Representing, as described Table 1, almost 68% of the calories consumed and an energy contribution from fat eight times greater than offered by SD. In contrast, the major energy source of SD-fed groups derived from high carbohydrates quantities, around 72% of calories weekly consumed. This significant contribution of energy from carbohydrates was seven times greater than in HFD and represents a substantial discrepancy in the average of consumed carbohydrates. In fact, while the groups HFD-fed presented a weekly consumption average of only 12.71 grams of carbohydrates, the groups administered with SD consumed 211.14 grams per week. The protein contribution was also conditioned by the average consumed diet. Animals SD-
fed consumed twice as much protein than HFD-fed rats, around 56.28 grams/week. However, the energetic contribution from protein content was almost insignificant between diets, just 2% higher in HFD. The average of macronutrients independent consumption is described in Figure 3.

![Figure 3](image)

**Figure 3.** Mean values (±Standard deviation) of weekly average macronutrients consumption in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups. (*** p<0.001 vs. SSD; **** p<0.0001 vs. SSD; #### p<0.0001 vs. SHFD; †††† p<0.0001 vs. ASD; ‡‡‡ p<0.001 vs. AHFD; ‡‡‡‡ p<0.0001 vs. AHFD).

### 3.3. Diet modulation and sedentarism effects on body weight, tibia length, and body mass variation.

The key results regarding the influence of diet and sedentarism on body weight, tibia length and percentage of body mass variation are described in Table 2. Despite the strong contrast in energy density and nutrients composition, the HFD was not able to induce significant differences in absolute body weight between groups during the 21 protocol weeks. However, animals surprisingly showed a relative body weight decrease when controlled for tibia length, declines of approximately 20% in sedentary and 16% in the active group HFD-fed when comparing to the SSD group (P <0.05 vs. SSD). Regarding tibia length, in absolute values, both HFD-fed groups had significantly longer tibias (P <0.05 vs. SSD). When normalized to body weight, the outcomes showed that sedentarism seems to have negatively modulated tibia length from sedentary groups animals independently of administered diet, as evidenced by the normalized tibia length to the body weight (Tab. 2). However, just SSD group show a statistic significance fronts the actives groups (P <0.05 vs. AHFD and ASD). The body weight behavior throughout the protocol and the final mean is also presented in Figure 4.
Table 2. Body Weight (BW) and Tibia Length (TL) characterization.

<table>
<thead>
<tr>
<th></th>
<th>SSD</th>
<th>SHFD</th>
<th>ASD</th>
<th>AHFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>373 ± 15.4</td>
<td>377.2 ± 14.51</td>
<td>369.7 ± 17.68</td>
<td>358.1 ± 16.43</td>
</tr>
<tr>
<td>BW/TL (g/cm)</td>
<td>119.3 ± 2.51</td>
<td>95.74 ± 3.46</td>
<td><strong>108.4 ± 3.52</strong></td>
<td>99.85 ± 5.47 **</td>
</tr>
<tr>
<td>TL (cm)</td>
<td>4.28 ± 0.1135</td>
<td>4.58 ± 0.240</td>
<td><em>4.37 ± 0.1947</em>*</td>
<td>4.54 ± 0.1813 *</td>
</tr>
<tr>
<td>TL/BW (cm/g)</td>
<td>0.01073 ± 0.00028</td>
<td>0.01135 ± 0.00059</td>
<td>0.01159 ± 0.00049 **</td>
<td>0.01174 ± 0.00046 **</td>
</tr>
</tbody>
</table>

Mean values (± Standard deviation) of Body Weight (BW) and Tibia Length (TL) in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups. (* p<0.05 vs. SSD; ** p<0.01 vs. SSD).

Figure 4. Variation of body weight mean values along the experimental protocol in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups.

Interesting results were also observed as soon the pBMV were associated between groups as described in Figure 5. When the isolated effect of sedentarism was analyzed through comparison between groups without nutritional modulation, the sedentary animals showed a negative BMV of approximately 3% compared to control group (P <0.05 vs. ASD). However, comparing the SHFD and AHFD groups, the association between sedentarism and fat diet shown unexpectedly to not be the most significant environmental condition in the modulation of pBMV.
presenting a lower and negative body mass variation of just 0.04% regarding the active group HFD-fed (P <0.05). When isolated, the effect of dietary modulation seems not to follow the trend presented by the association between fat diet and forced sedentary lifestyle. In fact, the comparison between the AHFD and ASD groups showed a positive variation of 8.62% in the AHFD group body mass, suggesting that the individual action of these factors seems to be more determinant in pBMV.

**Figure 5.** Mean values (±Standard deviation) of percentage of body mass variation (BMV, %) in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups. (* p<0.05 vs. SSD; ** p<0.01 vs. SSD; # p<0.05 vs. SHFD; † p<0.05 vs. ASD; ‡‡ p<0.01 vs. AHFD).

### 3.4. Morphometric features

The morphometric features related to the diet and physical inactivity effects are summarized in Table 3. Both HFD-fed groups presented a significantly higher relative and absolute retroperitoneal adipose tissue. An increase of 100% in SHFD and AHFD group when compared to the control group (P <0.05 vs. ASD). As expected, the ASD group presented the lowest fat retroperitoneal deposition (P <0.05 vs. SSD, SHFD, and AHFD). Diet manipulation also induced hepatic and pancreatic morphometric repercussions. We observed reductions in absolute and relative pancreatic weight in both HFD groups when compared to groups fed with standard diet (P <0.05 vs. SSD and ASD). Significant decreases in relative hepatic mass were also observed in the animals belonging to SHFD (P <0.05 vs. SSD and ASD) and AHFD group (P <0.05 vs. SSD). Even without any normalization, sedentary animals fed with fat diet also present a lower liver weight (P <0.05 vs. SSD). Results also reveal that the unbalanced diet and sedentarism was unable to induce significant morphometric changes in skeletal muscles as
demonstrated by absolute and relative weight of soleus and heart. However, gastrocnemius presents alterations in both relative and absolute weight. When normalized to tibia length, gastrocnemius shows the most significant alterations, especially in groups HFD-fed. Both, AHFD (P <0.05 vs. ASD) and SHFD (P <0.05 vs. SSD and ASD), presents a significant weight reduction. In fact, groups fed the same diet presented very similar values regarding gastrocnemius absolute and relative weight demonstrating, respectively, a mean of 15% and 20% of weight reduction.

Table 3. Morphometric features.

<table>
<thead>
<tr>
<th></th>
<th>SSD</th>
<th>SHFD</th>
<th>ASD</th>
<th>AHFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RATW (g)</td>
<td>14.87 ± 1.302</td>
<td>19.74 ± 1.903 ††</td>
<td>9.432 ± 1.611</td>
<td>18.99 ± 2.315 ††</td>
</tr>
<tr>
<td>RAT/TL (g/cm)</td>
<td>3.475 ± 0.952 †</td>
<td>4.326 ± 1.106 ††</td>
<td>2.131 ± 1.022</td>
<td>4.173 ± 1.304 ††</td>
</tr>
<tr>
<td>LW (g)</td>
<td>13.17 ± 0.43</td>
<td>11.01 ± 0.34 †</td>
<td>12.88 ± 0.41</td>
<td>11.59 ± 0.68</td>
</tr>
<tr>
<td>LW/TL (g/cm)</td>
<td>3.078 ± 0.335</td>
<td>2.412 ± 0.269 **,†</td>
<td>2.947 ± 0.267</td>
<td>2.554 ± 0.407 *</td>
</tr>
<tr>
<td>PW (g)</td>
<td>1.066 ± 0.1125</td>
<td>0.74 ± 0.0620 ††</td>
<td>1.196 ± 0.0603</td>
<td>0.711 ± 0.0803 ††</td>
</tr>
<tr>
<td>PW/TL (g/cm)</td>
<td>0.249 ± 0.082</td>
<td>0.161 ± 0.032 ††</td>
<td>0.273 ± 0.042</td>
<td>0.157 ± 0.048 ††</td>
</tr>
<tr>
<td>GW (g)</td>
<td>3.038 ± 0.138</td>
<td>2.595 ± 0.0191 †</td>
<td>3.069 ± 0.089</td>
<td>2.593 ± 0.109 *</td>
</tr>
<tr>
<td>GW/TL (g/cm)</td>
<td>0.7093 ± 0.095 ††</td>
<td>0.5676 ± 0.034 **,††</td>
<td>0.7031 ± 0.067</td>
<td>0.572 ± 0.070 ††</td>
</tr>
<tr>
<td>SW (g)</td>
<td>0.184 ± 0.006</td>
<td>0.1817 ± 0.014</td>
<td>0.198 ± 0.008</td>
<td>0.1971 ± 0.018</td>
</tr>
<tr>
<td>SW/TL (g/cm)</td>
<td>0.0429 ± 0.004</td>
<td>0.0324 ± 0.018</td>
<td>0.0452 ± 0.005</td>
<td>0.0435 ± 0.011</td>
</tr>
<tr>
<td>HW (g)</td>
<td>1.163 ± 0.055</td>
<td>1.095 ± 0.052</td>
<td>1.210 ± 037</td>
<td>1.296 ± 0.063</td>
</tr>
<tr>
<td>HW/TL (g/cm)</td>
<td>0.270 ± 0.040</td>
<td>0.239 ± 0.026</td>
<td>0.277 ± 0.028</td>
<td>0.286 ± 0.043</td>
</tr>
<tr>
<td>BrW (g)</td>
<td>2.059 ± 0.1238</td>
<td>2.083 ± 0.108</td>
<td>2.121 ± 0.077</td>
<td>2.106 ± 0.1075</td>
</tr>
<tr>
<td>Br/TL (g/cm)</td>
<td>0.476 ± 0.03362</td>
<td>0.4538 ± 0.03421</td>
<td>0.497 ± 0.02565</td>
<td>0.4645 ± 0.03552</td>
</tr>
</tbody>
</table>

RATW (retroperitoneal adipose tissue weight); LW (liver weight); PW (pancreas weight); GW (gastrocnemius weight); SW (soleus weight); HW (heart weight); Br (brain weight); BW (body weight); TL (tibia length). Mean values (±Standard deviation) of morphometric characteristics in SSD (Sedentary Standard Diet), SHFD (Sedentary High Fat Diet), ASD (Active Standard Diet), and AHFD (Active High Fat Diet) groups. (* p<0.05 vs. SSD; ** p<0.01 vs. SSD; † p<0.05 vs. ASD; †† p<0.01 vs. ASD; ‡‡ p<0.01 vs. AHFD).
4. Discussion

This study provides evidence that environmental factors influence growth and development processes, determining the adult phenotype of rodents. Using dietetic manipulation and imposing sedentarism since young ages, it was possible to observe different adult phenotypes involving eating pattern and morphological alterations such as animal’s dimensions and organs mass.

**Isolated diet effects on active animals.**

It is already known that eating habits play fundamental importance in animals’ growth and development processes and, perhaps, even determine the phenotype of an adult individual (Rezzi et al., 2007). The exposure to a dietary pattern since young ages, or even before birth, can condition metabolic (Benyshek, 2013; Hales & Barker, 1992; Hales & Barker, 2001) and morphological characteristics in adult ages (de Brito Alves et al., 2017). In our study, comparing both active groups (ASD vs. AHFD), it was possible to observe the sole diet effects on the animal’s adult phenotype. Namely, the long-term exposition to fat diet since young ages (4 weeks old), induced an unexpected decrease of food consumed. The mechanisms behind this behavior are still unknown, but Leonard and colleges (Leonhardt & Langhans, 2004) suggest that changes in hormone levels and increases in hepatic and other metabolic sensitive tissues related to high-fat consumption, probably play a crucial role. The similarity between both groups regarding caloric intake and the BW presented all over the protocol reflects this effect. Interestingly, the HFD ability to guide the eating pattern predisposing individuals to sugar and fat intake and also interfere with the mechanisms of satiety control (Haghshenas et al., 2014; Savastano & Covasa, 2005; Woods, Seeley, Rushing, D’Alessio, & Tso, 2003), was not observed as already described in the similar model (Tryon et al., 2015). However, it should be noted that most experimental studies with rodents antagonize the active nature of these animals by establishing sedentary rats as control groups. Mainly due to the relationship between physical activity and the control mechanisms of satiety and caloric consumption (J. Blundell, Gibbons, Caudwell, Finlayson, & Hopkins, 2015). Although the absence of CI and BW differences between ASD and AHFD, it
must be noted that the striking difference in dietary consumption seems to have affected the development process of the pancreas and gastrocnemius muscle, crucial tissues in energy balance (Jouvet & Estall, 2017; Schnyder & Handschin, 2015). Considering the importance of gastrocnemius for the animal ambulation, changes in this muscle weight reflect alterations in the entire skeletal muscle mass being a constant and current target in the characterization of muscular responses to different experimental and pathological contexts (De Jong et al., 2017; Keller et al., 2017). Once there was no difference in BW and CI between active groups, our results suggest that the AHFD group lower protein ingesting might be had conditioned the amount and availability of amino-acids for protein synthesis (Paddon-Jones et al., 2004), affecting the development of those organs negatively. As well, despite the apparent impairment of muscle mass, the HFD induced a significant retroperitoneal fat deposition explaining the maintenance of BW. Still, the fat diet has not influenced the animal growth as expressed by the non-existence of differences in tibia length and pBMV between ASD and AHFD. However, even with the lack of differences in tibial size, we do not have bone quality indicators, either related to protein content or bone mineral content.

**Isolated sedentary effects.**

It is widely accepted that sedentary behavior has been irrefutably associated with harmful effects on human health (Hind, 2016; Paul et al., 2016). However, when applied in young ages, there is no conclusive evidence about its repercussions on growth process and adult phenotype. To observe the isolated influence of sedentarism on adult animal’s phenotype, we have compared both SD-fed groups, SSD vs. ASD. The results revealed that sedentarism per se is not strong enough to influence eating behavior or BW as HFD did. These results are not in agreement with the evidence published in the last years, which associate sedentarism with increased FC, overweight and obesity in HFD animal models (Buettner, Schölmerich, & Bollheimer, 2007; Lu et al., 2016). However, in our results, although any difference was observed in the sedentary animal’s behavior related to FC and final absolute BW, the sedentarism induced an impressive retroperitoneal fat deposition, even without a variance of the energy intake. This interesting result
suggests, considering the irrefutable correlation between eating patterns, visceral obesity and predisposition to cardiovascular diseases (Mozaffarian, 2016; Sena, Pereira, Fernandes, Letra, & Seiça, 2017), that sedentarism can be a dangerous modulator of body composition, even without significant clinical manifestations such as overweight and obesity. As one of the three abdominal compartments of fat deposition, the retroperitoneal fat has been suggested as an important immunomodulator through its endocrine action, and a dangerous inducer of disorders in glucose and lipid metabolism through the fatty acid flow to the kidneys, pancreas and the vena cava (Hung et al., 2014; Tchernof & Després, 2013). As well, any significant difference regarding organs weight was observed, but the morphometric analysis shows that sedentarism affected the animal’s growth. In fact, the sedentary animals presented a smaller tibia when normalized to BW with a significant difference in the comparison. Probably, the local factors seem to be more determinant of bone growth and bone quality (Fonseca, Moreira-Gonçalves, Coriolano, & Duarte, 2014; Iravani, Lagerquist, Ohlsson, & Sävendahl, 2017; Yu, Suárez-González, Khalil, & Murphy, 2015). Considering the active nature of the rat (Garland Jr, Cadney, & Waterland, 2017), our results indicates that the sedentarism can play a more prominent role than diet in the animal’s growth and the adult phenotype.

**Combined effects of sedentarism and HFD.**

Through the comparison between the SHFD experimental group and the ASD control group, it was possible to evaluate the combined effects of the sedentarism and the fat diet. Following the eating patterns presented by the active group HFD-fed, the association between the sedentarism and the high-fat consumption induced a pattern of caloric and food consumption very similar to the AHFD group. Unexpectedly, the SHFD group had the lowest values of food consumption not only in comparison to the ASD group but among all groups. Based on the results presented by the active group HFD-fed, this pronounced effect induced by the HFD on FC seems to have been exacerbated by the imposed sedentary behavior. Consequently, the ASD control group presented a significantly higher FC compared to the sedentary animals HFD-fed. As well, as the results demonstrated by the isolated analyzes of the sedentarism and the fat diet, the SHFD
BW reflected the induced eating pattern with no significant differences regarding ASD group. Possibly, this outcome results from the counterbalancing effect between the amount of food consumed and the difference in energy density between the diets. In fact, these results interestingly contradict most previous studies which show that the combination of a sedentary lifestyle and hypercaloric/high-fat diets in adult animals leads, at least, to overweight and marked obesity [20, 28]. In addition to the proposed by Leonhardt et al. [6], our results suggest the activation of efficient mechanisms of energy control. The association between both HFD and sedentarism also presented controversial results regarding the morphometric and growth parameters. In agreement with the diet isolated effects, but not with the sedentarism, the association of these factors did not influence both morphometric and growth assessed variables. Even despite the long-term exposure, any statistical difference in BW, pBMV, and TL was observed in adult animals between SHFD vs. ASD. Given the pancreas and gastrocnemius weight loss in the AHFD group, these interesting results suggest that the combination of sedentarism with high-fat diet may have a compensatory action.

**Diet and physical activity interaction**

The animals physically active and fed with HFD presented higher levels of voluntary physical activity throughout the protocol. The high levels of VPA might explain the lower food consumption presented by the AFD compared to the animals fed with a standard diet. It is known that physical activity can induce adjustments in the control mechanisms of food intake and satiety such as alterations in gastrointestinal hormone response, gastric emptying, and in metabolism and endocrine response of muscle and adipose tissue (J. Blundell et al., 2015). Indeed, changes in the endocrine response involved in satiety, such as leptin, ghrelin, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), and adaptations in energy metabolism such as alteration in substrate oxidation in muscle and liver, have been suggested as possible mechanisms of physical activity in the control of satiety and food intake (Farooqi et al., 2007; Hopkins, Jeukendrup, King, & Blundell, 2011; Stensel, 2010). Physical activity also influences the central control of satiety and food consumption through hedonistic, rewarding and dopaminergic pathways. Hormones such as
leptin can inhibit mesolimbic dopamine neurons and suppress food intake, while ghrelin activate dopaminergic neurons in the ventral tegmental area, increasing the dopamine turnover in the nucleus accumbens and stimulating food intake (Carhuatanta et al., 2011; Hopkins et al., 2011; Mani et al., 2018). The results regarding physical activity and food consumption presented by the AFD support the evidence related control of the satiety and the dietary intake by the physical activity. However, the evidence regarding controlling mechanisms of food consumption is still poorly understood and conflicting, and the possible mechanisms to explain the low food and caloric intake presented by the SFD group, seem to be different. While the mechanisms related to physical activity adopt a psychobiological approach to explain appetite and food consumption control where physiological mediators act as conductors of behavior, the lipostatic approach where metabolic fuels induce behavioral properties seeking for energy balance has been suggest explaining fat intake influence in this mechanisms (J. Blundell et al., 2015; J. E. Blundell, 2002; Leonhardt & Langhans, 2004). Namely, disturbances on fatty acid oxidation due to excessive fat consumption may influence energy consumption through the stimulation of vagal afferent nerve activity due to changes in the hepatic but also in intestinal enterocytes ratio of ATP / ADP seems to be an essential mechanism (Friedman, 1995; Langhans, 2009).

Although our work aimed a general assessment of the effects of sedentarism and high fat consumption on the adult rodent phenotype, the lack of histological and functional evaluations may be a limitation of our study, particularly due to the weight changes presented by some evaluated organs and by the lack of differences in BW between groups.

5. Conclusion

In summary, our results suggest that the manipulation of diet and physical activity levels in early ages, conditionate the adult phenotype in a growing animal model. Interestingly, the animals HFD-fed presented an unexpected phenotype, being larger and lighter with lighter organs
than animal’s treated with SD and, even without weight gain, they present an expressive retroperitoneal fat deposition, more notorious in sedentary individuals.

6. Acknowledgments

The authors also thank for the support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasil). This study was financed by the Fundação para a Ciência e Tecnologia (FCT/UID/DTP/00617/2019).
7. Bibliography


STUDY 2

Antonio Bovolini, Nádia Gonçalves, Juliana Garcia, Maria A. Andrade, José A. Duarte (2019). *Relative role of fat diet and physical inactivity to metabolic syndrome and non-alcoholic fatty liver disease development in Wistar rats.*

Submitted to *Nutrition, Metabolism & Cardiovascular Diseases.*
Relative role of fat diet and physical inactivity to metabolic syndrome and non-alcoholic fat liver disease development in Wistar rats

Antonio Bovolini a*, Nádia Gonçalves b, Juliana Garcia c, Maria A. Andrade d, José A. Duarte a*

a CIAFEL - Laboratory of Biochemistry and Experimental Morphology, Faculty of Sport, University of Porto; R. Dr. Plácido da Costa 91, 4200-450 Porto, Porto, Portugal.

b Department of Surgery and Physiology, Faculty of Medicine, University of Porto; Rua Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal.

c UCIBIO / REQUI - Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto; R. Jorge de Viterbo Ferreira 228, 4050-313 Porto, Portugal.

d Federal University of Pernambuco; Av. Prof. Moraes Rego, 1235 - University City, Recife - Pernambuco, 50670-901, Brazil.

* Authors to whom correspondence should be addressed (+351 918732526; e-mail: jabovolini@hotmail.com and jarduarte@fade.up.pt)
Abstract

**Aim:** This study aimed to analyze the relative contribution of fat diet (FD) and physical inactivity (PIn) to the development of metabolic syndrome (MS) and non-alcoholic fat liver disease (NAFLD). **Methods:** Male Wistar rats were randomly divided into 4 groups (n=10/group): active groups, fed with fat (AFD) or standard (ASD) diet; and physically inactive groups, fed with fat (SFD) or standard (SSD) diet for 21 weeks. Plasmatic fasting levels of triglycerides, total cholesterol, glucose, as well as glucose tolerance and insulin resistance were evaluated before sacrifice for MS diagnosis. For liver morphological analysis, the collagen content, cell apoptosis, nuclear NF-kB activation, macrophages phenotype and NAFLD activity score were evaluated. **Results:** Animals from SFD and AFD groups were all diagnosed with MS. Contrasting to the normal liver structure of AFD, SFD showed a diffuse and intense microvesicular steatosis, with enhanced number of inflammatory cells infiltration (M1 macrophages), hepatocytes with NF-kB activation and signs of apoptosis, as well as a spread interstitial collagen content. These signals were more tenuous in AFD, with higher amount of M2 macrophages and punctual macrovesicular steatosis. Comparing to both active groups, SSD presented elevated levels of M1 macrophages and hepatocytes with NF-kB activation or apoptosis without steatosis and interstitial fibrosis. **Conclusion:** FD and PIn showed harmful effects on metabolic parameters and histological liver profile, especially when combined. FD was more determinant than PIn in inducing NAFLD and MS, however PIn boosted the harmful effects of FD through the modulation of liver inflammatory response.

**Keywords:** lifestyle, metabolic disturbances, liver histology, NAFLD, Inflammation, Sedentarism.
Introduction

Lifestyle has been recognized as a determinant factor in the epidemic of non-communicable diseases (NCDs) such as hypertension, type 2 diabetes mellitus (T2D), and obesity [1]. As a critical factor, the current and globalized food pattern based on highly processed and caloric foods containing high concentrations of sugar and fat, are undeniably linked to the cluster of metabolic syndrome’s (MS) disturbances [2]. Among them, liver disorders are early manifestations and an important sensor of metabolic balance [3], highly connected to obesity, IR, and MS, being the non-alcoholic fatty liver disease (NAFLD) the predominant hepatic disorder worldwide [4]. The role of diet in the development of obesity-related chronic diseases, including NAFLD and MS, has been a focal point in literature [2, 5]. This spotlight is shared with the important therapeutic role of physical activity in chronic diseases related to lifestyle [6-8]. However, the role of physical inactivity (PIn) as an independent pathogenic factor for metabolic diseases has been in the background even with the alerts of World Health Organization (WHO) showing that sedentary lifestyle and insufficient physical activity are linked to 19.5 million annual deaths due to NCDs [9]. Indeed, the current literature does not provide a clear picture about the impact of PIn on NCDs, such as MS and NAFLD.

Moreover, considering the beneficial role of regular physical activity in the treatment of NAFLD, PIn has been suggested as a pathogenic factor of NAFLD, especially when associated with obesity [10-12]. However, assuming fat diet and PIn as main lifestyle threats leading to NCDs, it is not known the responsibility of each one as well as their potential interactions to the development of NAFLD. Considering this, the present study aimed to analyze the effect of PIn and fat diet, isolated or conjugated with each other, on the development of MS and hepatic histologic profile in an animal model. Knowing that imposed physical exercise programs might induce significant physiologic stress to animals leading to maladaptations, since these protocols do not mimic the normal routine of intermittent rodent activity in the wild [13], for this study it was chosen a voluntary exercise protocol for the active control animals (standard or fat diet fed), allowing their free access to a running wheel. Rats without access to the running wheel, which were confined to their small cage space, constituted the experimental physical inactive animals (standard or fat diet fed). We hypothesize that PIn and fat diet are both independent modulating factors, predisposing animals to MS and to liver alterations related to NAFLD.
Material and methods

Animals

Male Wistar rats, 4 weeks old (183.55±3.48g), were purchased from Charles-River (Barcelona, Spain) and kept at room temperature (22°C), with relative humidity of 60 ± 10% and inverted 12h light/dark cycles for 22 weeks. The study was approved by the local ethical committee and the animals’ housing and experimental treatment were in accordance with the guidelines defined by the European Council Directive (2010/63/EU) transposed into Portuguese law (Decreto-Lei n.º 113/2013, de 7 de Agosto).

Study design

After 1-week of acclimatization fed with a standard diet (SD), the animals were randomly divided into four groups (n =10/group): active groups, fed with fat (AFD, experimental diet group) or standard (ASD, the control group) diet; and physically inactive groups, fed with fat (SFD, experimental diet+physical inactive group) or standard diet (SSD, experimental physical inactive group). The length of the experimental protocol was 21 weeks.

Diet

During the 21-week experimental protocol, the animals were fed a standard diet (A04) or high-fat diet (diet purified HF 231), both from Scientific Animal Food & Engineering (SAFE, France). The animals had food and water access ad libitum and the food intake were recorded weekly in a precision balance (Kern 870, Balingen, Germany) for caloric and macronutrients consumption assessment. Diet composition was provided by the manufacturer (SAFE, France). Diet description is described in Table 1.

Table 1. Diet characterization.

<table>
<thead>
<tr>
<th></th>
<th>Standard Diet</th>
<th>High-Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC</td>
<td>ED (Kcal)</td>
</tr>
<tr>
<td>Fat</td>
<td>3.1%</td>
<td>8.4% (279)</td>
</tr>
<tr>
<td>Protein</td>
<td>16.1%</td>
<td>19.3% (644)</td>
</tr>
</tbody>
</table>
Carbohydrate 60.4% 72.4% (2416) 14.2% 10.6% (568)

Percentages of macronutrients composition (MC) and energy density (ED). Standard Diet (3.339,00 Kcal/Kg); High-Fat Diet (5.335,80 Kcal/Kg). Data regarding energy contribution and diet composition were provided by SAFE (France).

Voluntary physical activity (VPA) and imposed physical inactivity (PIn)

The animals from physically active groups (ASD, AFT) were individually housed in activity wheel and distance counter equipped cages, where they had free access to the activity-wheel. The total running distance along the protocol was used to characterize the physical activity levels of active groups. The animals belonging to the physically inactive groups were individually housed in cages with no activity-wheel, with their daily physical activity restricted to the cage space. Both cages had an identical floor area (800 cm²; Tecniplast, Buguggiate, Italy).

Metabolic and blood pressure evaluation

The metabolic tests, namely glucose tolerance, triglycerides (TGC), and total cholesterol (TC) were performed three days before the animals’ sacrifice using blood from the tail. After 6h fasting, the TGC and TC were measured at baseline. For the glucose tolerance test, glycemia was measured at baseline, and 15, 30, 60, 90, and 120 min after administration of 1g/kg of glucose by gavage. Two days before the animals’ sacrifice and after a fasting period of 6h, the resting blood pressure and the insulin resistance were assessed. The blood pressure was determined using an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT) as described before [14, 15]. The animals were placed into an animal holder 15 min before the blood pressure assessment to prevent undue stress. Blood pressure was measured during 10-minute sessions until we obtained 3 stable recordings. Insulin resistance was tested according to the methodology described elsewhere through the measurement of glycemia at baseline, and 15, 30, 60, 90, and 120 min after 0.5 U/kg intraperitoneal insulin injection. Blood was collected from the tail vein by puncture with a 25-gauge needle. Glycemia and lipid profile were measured using Cholestech LDX Lipid Profile Glucose cassettes by the Cholestech LDX System (Cholestech Corporation, Hayward, CA) following the manufacturer’s instructions.
Metabolic syndrome assessment

Following the World Health Organization definition for human criteria (WHO) [16], animals were diagnosed with metabolic syndrome in the presence of impaired glucose tolerance (IGT) or insulin resistance (IR) with the concomitant presence of two or more risk factors such as enhanced central obesity, elevated blood pressure, and dyslipidemia (high TGC and TC levels). The existence of any of these risk factors in each animal was considered for values above the percentile 100 of the control group (ASD).

Animal sacrifice and samples collection

Following the 21-weeks experimental period, animals were weighed, anesthetized with an intraperitoneal injection of Ketamine (90mg/kg, Merial, France) and Xylazine (10mg/kg, Bayer, German), and euthanized by exsanguination. Then, the retroperitoneal adipose tissue from both sides (RAT) and liver were harvested, washed in PBS (pH 7.2), weighed in a precision balance (resolution 0.01mg; Kern 870, Balingen, Germany), and samples from all hepatic lobes were processed immediately for light microscopy. Relative liver weights (rLW) were calculated by the respective ratio for body weight (BW) and expressed as percentage (%). Values above the percentile 100 of RAT absolute weight registered in ASD group were used to define central obesity.

Histology

Tissue processing for light microscopy: liver samples were fixed for 24h in 4% paraformaldehyde solution at 4°C, dehydrated through graded ethanol solutions, cleared in xylene and mounted in paraffin. 5µm thick sections from all lobes were cut and used for assessing collagen tissue content, NAFLD Activity Score (NAS), macrophage polarization (M1 and M2 activation), nuclear factor kappa B (NF-kB) expression and apoptotic cells count. Sections were analyzed with a light microscope (Axio Imager A1, Carl Zeiss; Germany) and images recorded with a coupled digital camera (Leica DM4000B, Nussloch, Germany).

Collagen tissue content assessment: liver sections were stained with Pricosirius Red according to the method of Sweet et al. [17] by incubation on 0.1% Sirius red picric acid for 90 min. Then, sections were rinsed in 0.5% acetic acid, dehydrated in ethanol and cleared in xylene.
Collagen and the hepatocytes were stained in light red and yellow, respectively. Image-Pro Plus 6.0 (Media Cybernetics, Inc) was used to quantify the percentage red and yellow area.

**Apoptosis assessment:** terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) technic was used to assay the presence of liver apoptotic nuclei with a commercial kit following the manufacturer instructions (*In situ cell death detection kit AP*, Cat. 11684809910, Roche). After routine deparaffinization, sections were immersed in 0.1M citrate buffer (pH 6.0) and submitted to antigen retrieval in pressure cooker for 10 min. After cooling at room temperature immersed in 0.1M citrate buffer, sections were rinsed in PBS and blocked with 3% bovine serum albumin (BSA) solution in PBS for 30 min at room temperature and, then, incubated in freshly prepared TUNEL nucleotide mixture in a humidified and dark chamber at 37°C for 60 min followed by the incubation with converter-AP during 30 minutes at 37°C. Negative and positive controls were simultaneously prepared by incubation with label solution only (nucleotide mixture) or by incubation with deoxyribonuclease I (DNase I, cat. 10104159001, Sigma-Aldrich) before the labeling procedure, respectively. Sections were then washed under gentle stirring and incubated with 4-Chloro-2-methylbenzenediazonium/3-Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate (SIGMAFAST™ FAST RED, cat. F4648-50SET, Sigma-Aldrich) reagent for 5 minutes. After washing, slides were counterstained with a solution of hematoxylin-water (dilution, 1:10) for 3 min. The number of TUNEL nuclear staining was expressed as nuclei/µm².

**NF-κB and macrophage polarization assessment by immunohistochemistry:** liver deparaffinized sections were rinsed in distilled water and incubated in PBS for 10 min before the antigen retrieval in a pressure cooker for 10 min in 0.1M citrate buffer (pH 6.0). After refrigerated at room temperature immersed in 0.1M citrate buffer, sections were washed twice (5 min each) with PBS solution and the endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min. Non-specific binding sites were blocked with 3% BSA in PBS for 30 min. Each slide was them incubated with anti-NF-κB p50 polyclonal rabbit antibody (diluted 1:50; sc114, Sta. Cruz), Anti-CD68 polyclonal rabbit antibody (diluted 1:100; ab125212, Abcam) for M1 macrophage quantification and anti-Mannose receptor rabbit polyclonal antibody (diluted 1:100; ab64693, Abcam) for M2 macrophage quantification in PBS-T overnight (4°C).Sections were washed with PBS and then incubated with a goat anti-rabbit IgG peroxidase conjugated secondary antibody (diluted 1:100; Ab97051, Abcam) in PBS-T for 2 h at 37 °C (the same
secondary antibody was used for all labeling). Sections were then washed under gentle stirring and incubated with 3,3’-diaminobenzidine tetrahydrochloride (SIGMAFAST™ DAB, cat D4293-50SET, Sigma-Aldrich) reagent for 5 minutes. After the reaction interruption and subsequent washing, slides were counterstained with diluted hematoxylin-water (1:10) for 3 min. Negative controls were performed with the omission of the primary antibody incubation step. Six microscopic visual field per section/lobe were evaluated per animal. Results were expressed as the number of positive cells per section.

**NAFLD Activity Score (NAS) assessment:** liver sections were routinely stained with hematoxylin-eosin (H&E). Light microscopy analysis findings were scored using the NASH Clinical Research Network Scoring System [18]. The NAFLD activity score (NAS) was defined as the unweighted sum of scores for steatosis, lobular inflammation and hepatocyte ballooning as detailed in Table 2. A score equal or lower than 2 was considered not diagnostic of steatohepatitis according to the **NAFLD Activity Score [18]**.

**Table 2. Rank for Non-Alcoholic Fat Liver Disease activity score (NAS score).**

<table>
<thead>
<tr>
<th>NAS</th>
<th>Steatosis</th>
<th>Ballooning</th>
<th>Lobular Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;5% (0)</td>
<td>None (0)</td>
<td>None (0)</td>
</tr>
<tr>
<td>3</td>
<td>5-33% (1)</td>
<td>Rare or few (1)</td>
<td>1-1 foci / 20 x field (1)</td>
</tr>
<tr>
<td>6</td>
<td>34-66% (2)</td>
<td>Many (2)</td>
<td>2-4 foci / 20x field (2)</td>
</tr>
<tr>
<td>8</td>
<td>&gt;66% (3)</td>
<td>Many (3)</td>
<td>&gt; 4 foci / 20x field (3)</td>
</tr>
</tbody>
</table>

Parentheses numbers represent the intensity’s score from histological characteristic. Steatosis quantification is defined by the percentage of area covered by fat vacuoles. The inflammatory assessment includes all types of inflammatory cells in the hepatic lobe. Extracted from [18].

**Statistical analysis**

The statistical analysis was performed using GraphPad Prism® (version 7.00, GraphPad Software, San Diego, California, USA). The Kolmogorov-Smirnov test was performed to investigate the data normality. The one-way ANOVA followed by the Tukey post hoc comparison test was used to analyze data with normal distribution. The remaining variables with abnormal distribution (NAFLD Activity Score, Collagen content, NF-kB activation) were analyzed with
Kruskal-Wallis followed by the Dunn’s post hoc comparison test. Once the existence of normal distribution of the data the differences among active groups was compared with two-tailed unpaired T-test. Differences were considered significant at p<0.05, and the obtained data were expressed as a mean ± standard deviation for normal distributed data or as median with percentiles 25 and 75 (P25-P75) for abnormally distributed data.

Results

The total physical activity levels were significantly lower (p<0.0001) in ASD group (12.77±885) compared to AFD (80.77±32.77), as described in Figure 1. Body weight and liver absolute and relative weight, after 21-weeks of experimental protocol, are depicted in Table 3. Experimental SSD and SFD groups presented no significant differences in BW comparing to ASD and AFD control groups. Liver weight (LW) from the FD-fed groups, AFD and SFD, was lower compared SSD and ASD. In what concern to relative liver weight (rLW), no significant differences were found between groups.

Table 3. Morphological characterization of body weight, liver absolute weight and relative weight.

<table>
<thead>
<tr>
<th></th>
<th>BW</th>
<th>LW</th>
<th>rLW</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSD</td>
<td>373.0±70.57</td>
<td>13.2±1.38</td>
<td>2.58±0.24</td>
</tr>
<tr>
<td>ASD</td>
<td>369.7±81.00</td>
<td>12.9±1.31</td>
<td>2.74±0.33</td>
</tr>
<tr>
<td>SFD</td>
<td>377.2±66.51</td>
<td>11.0±0.85</td>
<td>2.52±0.12</td>
</tr>
<tr>
<td>AFD</td>
<td>358.1±75.31</td>
<td>11.6±1.81</td>
<td>2.57±0.34</td>
</tr>
</tbody>
</table>

Body weight (BW, g), liver absolute weight (LW, g) and liver relative weight (rLW, %) from physically inactive standard diet-fed (SSD), active standard diet-fed (ASD), physically inactive fat-diet fed (SFD) and active fat-diet fed (AFD) groups. The results are expressed as means ± standard deviation.

η - p<0.05 vs. SFD;
δ - p<0.05 vs. AFD.
As presented in Table 4, all the animals from AFD and SFD groups presented the standards to diagnose MS according to WHO, exhibiting concomitant insulin and glucose resistance in addition to central obesity, dyslipidemia, and high blood pressure, fulfilling the adopted metabolic syndrome criterium (Data not shown). None of the animals from SSD fulfill the MS diagnose criteria (Data not shown).

Table 4. Occurrence of metabolic syndrome among groups.

<table>
<thead>
<tr>
<th></th>
<th>SSD</th>
<th>SFD</th>
<th>ASD</th>
<th>AFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Without MS</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Relative frequency (%) of animal hit by Metabolic Syndrome (MS) in physically inactive standard diet-fed (SSD), physically inactive fat-diet fed (SFD) and active fat-diet fed (AFD) groups, based on data of active standard diet-fed (ASD, control group).

NAFLD Activity Score

Light microscopy analysis from FD-fed groups indicated an intense and diffused micro-vesicular vacuolization suggestive of steatosis in SFD, contrasting with the punctual areas of fat
moderate macro-vesicular deposition observed in AFD (Figure 2). However, according to the NAS ranking [18] only the SFD group accomplished the NAFLD criterium. SFD animals presented pronounced hepatic fat deposition in hepatic zones 1 and 2, and significantly higher incidence of ballooned cells compared to SSD, ASD, and AFD animals. No significant differences were found in the number of inflammatory cells between groups. SSD and ASD groups presented similar histological steatosis score, lobular inflammation, and balloon cells, without significative differences between them. The statistical analysis (Table 5) showed a significant and pronounced difference between SFD and the remaining groups.

Table 5. NAFLD score components.

<table>
<thead>
<tr>
<th></th>
<th>SSD</th>
<th>SFD</th>
<th>ASD</th>
<th>AFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAS</td>
<td>0.04918±0.780</td>
<td>5.357±1.54</td>
<td>0±0</td>
<td>2.319±1.357</td>
</tr>
<tr>
<td>Steatosis</td>
<td>0±0</td>
<td>2.163±0.927</td>
<td>0±0</td>
<td>0.725±0.708</td>
</tr>
<tr>
<td>Ballooning</td>
<td>0.04918±0.218</td>
<td>1.633±0.581</td>
<td>0±0</td>
<td>0.4375±0.5342</td>
</tr>
<tr>
<td>Lobular Inflammation</td>
<td>0±0</td>
<td>1.561±0.538</td>
<td>0±0</td>
<td>1.156±0.520</td>
</tr>
</tbody>
</table>

Mean ± Standard Deviation of NAS score variables in all studied groups: physically inactive standard diet-fed (SSD), active standard diet-fed (ASD), physically inactive fat-diet fed (SFD) and active fat-diet fed (AFD) groups.

\(\pi p<0.0001\) vs. SFD;
\(\eta p<0.001\) vs. SFD;
\(\eta\eta p<0.0001\) vs. SFD;
\(\delta p<0.001\) vs. ASD;
\(\delta\delta p<0.0001\) vs. ASD;
\(\omega p<0.01\) vs. AFD;
\(\omega\omega p<0.0001\) vs. AFD).
**NAFLD score**

![Figure 2 - Light micrographs of liver sections stained with haematoxilin-eosin, representative of all studied groups: physically inactive standard diet-fed (SSD, a), active standard diet-fed (ASD, b), physically inactive fat-diet fed (SFD, c), and active fat-diet fed (AFD, d) groups. Macrolvesicular (yellow arrows) and microvesicular steatosis (black arrows), as well as ballooned cells (blue arrows) are depicted in c and d. The box-plot graphic of non-alcoholic fat liver disease (NAFLD) score is illustrated in e.](image)

\[ \pi p<0.0001 \text{ vs. SSD; } \delta p<0.0001 \text{ vs. ASD; } \omega p<0.0001 \text{ vs. AFD.} \]

**Histological appreciation of hepatic tissue structure**

Liver tissue structure from active groups showed curious results regarding histological appreciation. The ASD group presented, in general, a preserved tissue architecture without any evidence of cellular damage, cytoplasmic vacuolization suggestive of fat deposition, inflammatory cells infiltration or the occurrence of ballooned cells, presenting however slight signs of vascular congestion with sinusoidal dilatation (Fig. 2b). In contrast, the AFD group demonstrated architectural tissue alterations such as a moderate presence of micro and macro vesicles suggestive of hepatic steatosis and punctual presence of ballooned hepatocytes (Fig. 2d). Overall, in agreement with ASD, the SSD group also exhibited a well-preserved liver structure with no signals of architectural disorders (Fig. 2a). Nevertheless, some activated Kupffer cells are observed in SSD group (Fig. 4a). In comparison to AFD, the animals from SFD revealed pronounced pathological changes in hepatic tissue structure, with intense and diffuse microvesicular deposition, a marked number of ballooned cells and signals of vascular congestion with slight sinusoidal dilatation (Fig. 4c). In sedentary animals (SFD and SSD) the liver M1
macrophage phenotype was more frequent compared to the M2 phenotype, whereas in active animals (AFD and ASD) the M2 phenotype was predominantly expressed.

**Hepatic collagen fibers accumulation**

Hepatic collagen content is labelled in Figure 3. The SD-fed groups presented the lowest percentage of collagen content among all groups. Namely, the SSD presented a median of 1.813% (11.35%-2.845%) and the ASD 0.7993% (0.5626%-1.394%). Moreover, the SFD group presented a significant increase in collagen content compared to SSD (p<0.0001), ASD (p<0.0001) and AFD (p<0.01) animals, with a median of 19.27% (16.21%-27.15%). Despite showing a lower median value than SFD, the AFD group presented a significantly higher collagen content than SD-fed animals (p<0.01), with a median of 6.032% (3.868%-9.995%).

![Figure 3](image)

**Figure 3** – Hepatic light micrographs, stained with picrosirius red, representative of the studied groups: physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d). The median values (P25-P75 and the extreme values) of hepatic expression of collagen in all groups are depicted in e.

- $\pi$ p<0.01 vs. SSD;
- $\pi\pi$ p<0.0001 vs. SSD;
- $\delta$ p<0.01 vs. ASD;
- $\delta\delta$ p<0.0001 vs. ASD;
- $\omega$ p<0.01 vs. AFD.
Apoptosis

Evaluation of liver apoptotic cells is presented in Figure 4. Physically inactive groups (Fig. 4a and 4b) presented a higher number of hepatocytes with stained nuclei comparatively to the physically active groups (Fig. 4c and 4d). The comparison between groups fed with the same diet showed a significant difference (p<0.001) in the number of stained nuclei between the SSD and ASD (94.67±6.282 nuclei/µm² vs. 23±12.07 nuclei/µm², respectively) and between SFD and AFD (159.5±6.535 nuclei/µm² vs. 13.5±10.37 nuclei/µm², respectively) (Fig. 4e). The lack of differences between active groups also suggest an independent influence of physical inactivity on hepatocyte apoptosis.

Figure 4 - Light micrographs of liver sections representative of all studied groups, processed with terminal deoxynucleotidyl transferase dUTP nick and labeling method: physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d). Mean values ± standard deviation of nuclei/µm² are depicted in e. Stained hepatocyte nuclei are indicated by yellow arrows.

\( \pi \) p<0.01 vs. SSD;
\( \delta \) p<0.0001 vs. ASD;
\( \omega \) p<0.0001 vs. AFD.

NF-κB activation and macrophage polarization

The assessment of macrophage phenotype is depicted in Figure 5. The representative images from SSD (Fig. 5a) and SFD (Fig. 5b) groups show the role physical inactivity in hepatic proinflammatory M1 macrophage phenotype. Both inactive SSD and SFD groups displayed a significant higher number of stained CD68 cells in comparison to active groups ASD and AFD.
(Fig. 5c and 5d, respectively). This marked influence of physical inactivity can also be seen in M2 phenotype macrophages population in SSD and SFD (Fig. 5e and 5f, respectively), showing significantly lower values than ASD and AFD (Fig. 5g and 5h, respectively). Besides the pronounced difference presented by physically inactive groups related to liver M1 and M2 positive cells, the lack of significant differences in both markers between ASD and AFD groups, characterized in Table 6, emphasizes negative impact of physical inactivity on hepatic macrophage resident population.

Results regarding hepatic NF-κB activation, detected by stained nuclei, are also described in Table 6 and Figure 6. SSD and SFD expressed a significantly higher number of NF-κB positive nuclei in contrast to ASD and AFD groups. These pronounced differences suggest a determinant role of physical inactivity in hepatic NK-κB activation. The quantitative analysis of hepatic NF-κB activation is represented in Figure 6e, showing the significant differences between the inactive and active groups regardless of the used diet.

Table 6. Macrophage phenotype and NF-κB activation assessment

<table>
<thead>
<tr>
<th></th>
<th>SSD</th>
<th>SFD</th>
<th>ASD</th>
<th>AFD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M1</strong></td>
<td>20.1±8.89</td>
<td>62.3±26.03</td>
<td>6.498±3.17</td>
<td>9±3.59</td>
</tr>
<tr>
<td><strong>M2</strong></td>
<td>4.1±2.76</td>
<td>7.767±3.98</td>
<td>20.9±6.92</td>
<td>43.57±13.85</td>
</tr>
<tr>
<td><strong>NF-κB</strong></td>
<td>11.5 (9.25-15)</td>
<td>24 (22.75-26.25)</td>
<td>2 (1-3)</td>
<td>6 (3.75-9.25)</td>
</tr>
</tbody>
</table>

Mean ± Standard Deviation of cells/µm² from M1 and M2 and median values (P25-P75) of nuclei/µm² from NF-κB assessment in all studied groups: physically inactive standard diet-fed (SSD), physically inactive fat-diet fed (SFD), active standard diet-fed (ASD), and active fat-diet fed (AFD).

η p<0.001 vs. SFD;
δ p<0.05 vs. ASD;
δδ p<0.001 vs. ASD;
ω p<0.05 vs. AFD;
ωω p<0.0001 vs. AFD).
Figure 5 - Light micrographs of liver sections immunostaining for M1 (CD 68) and M2 (Mannose receptor) macrophage phenotypes, representative of all studied groups: physically inactive standard diet-fed (SSD, a and e), physically inactive fat-diet fed (SFD, b and f), active standard diet-fed (ASD, c and g), and active fat-diet fed (AFD, d and h). Mean values ± standard deviation of cells/µm² of M1 and M2 are depicted in i and j, respectively.

π p<0.0001 vs. SSD;
δ p<0.01 vs. ASD;
δδ p<0.0001 vs. ASD;
η p<0.0001 vs. SFD;
ω p<0.05 vs. AFD;
ωω p<0.0001 vs. AFD
Figure 6 – Light micrographs of liver sections immunostaining for NF-κB, representative of all studied groups: physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d). The median values (25thP-75thP) are depicted in e.

π p<0.01 vs. SSD;
δ p<0.05 vs. ASD;
δδ p<0.0001 vs. ASD;
ω p<0.05 vs. AFD;
ωω p<0.0001 vs. AFD.

Discussion

Results demonstrate the long-term harmful effects of physical inactivity and fat-diet intake on the metabolic syndrome development with non-alcoholic fat liver disease (NAFLD), as documented by the histological alterations after 21 weeks of experimental protocol. The histological changes found in the liver of fat diet-fed animals, such as the increased pro-inflammatory cells population (M1 macrophage phenotype), high apoptotic cells number, enhancement of collagen content and intense vacuolization suggestive of hepatic steatosis are similar to those recently described in the literature [19] although the lack of differences in body weight between fat diet-fed and standard diet-fed animals in our experimental design. Despite the absence of structural damage signals in standard diet-fed animals, it was observed that the histological alterations induced by fat diet were clearly aggravated in physically inactive animals, suggesting that physically inactivity has a boosting effect for NAFLD and MS development.

After ending the protocol, both FD-fed groups presented the criteria for MS diagnosis according to WHO [16]. Comparatively to the percentile 100 from ASD group results, used to define values above as abnormal, the SFD and AFD exhibited impaired glucose tolerance and
insulin resistance concomitantly with the presence of central obesity, dyslipidemia and elevated blood pressure. Given the comparison outcomes between FD and SD-fed animals, our results show that FD comparatively to PIn played a predominant role in the syndrome and syndrome-related NAFLD development since just FD-fed groups filled the adopted criteria for MS diagnosis. The existence of different NAFLD degrees between FD-fed groups also reinforce the concept that PIn is an aggravating factor, since the chronic fat-diet consumption has been implicated as an etiological factor of both MS and NAFLD [20, 21].

Even so, despite the alterations in liver architecture presented by FD-fed groups are similar to those already described in the literature, our results contrast with the predominant animal models of diet-induced NAFLD since no alterations were observed in the animals BW and most evidence related to hepatic disorders of this nature is linked to human and animal obesity or alcohol consumption [22-26]. On the other hand, the pronounced steatosis presented by the FD-fed animals compared to SD-fed groups is consistent with the role of lipid overload and NAFLD development [27, 28]. The free fatty acid (FFA) provided by diet but also from lipogenesis affects the lipid metabolism through protein kinase B (Akt) activity, leading to hepatic IR and ectopic fat deposition [29], which can explain the pronounced steatosis-like vacuolization found in FD-fed groups, especially in physically inactive animals. The mechanisms of liver adaptation to chronic PIn, which explain the different degrees of NAFLD in AFD and SFD, are still poorly known because the results from human studies on this topic mostly came from epidemiological follow-ups, while animals studies are focused on the influence of exercise and physical training and not on the effect of PIn on NALFD [30-32]. Even so, despite the undeniable link between PIn and metabolic disorders related to NAFLD development such as IR and central adiposity, as presented by SFD group [30], the underlying pathological mechanisms remain unknown. Disturbances in the elimination of fatty acids-related metabolites or hepatic oxidative pathways have been proposed as a pathogenic component of NAFLD-related steatosis-like vacuolization under PIn conditions [33]. The decreased liver mitochondrial content with reduced organelle functioning promoted by PIn, supports the concept that a reduced hepatic oxidative capacity might turn the liver more susceptible to insults such as lipid overload, justifying the pronounced steatosis-like vacuolization presented by the physically inactive animals compared to the active rats in the FD-fed groups [34]. Our results also suggest that comparatively to physical inactivity,
the diet’s influence appear to be predominant in hepatic fat deposition and consequently in steatosis progress. Considering that among FD-fed groups the steatosis-like vacuolization presence was expressively higher in the physically inactive animals, our results seem to corroborate with the theory that the Pln might not be, per se, determinant to induce hepatic disorders but, in fact, it seems to be a major enhancer of structural changes induced by lipid overload.

Differently to the observed primer influence of fat diet on liver steatosis, the physically inactive groups, independently of the diet, presented a pronounced inflammatory hepatic profile when compared to physically active groups, with a high number of hepatocyte nuclei stained for NF-κB and predominance of the M1 macrophage phenotype. These results manifested by both physically inactive animals from SSD and SFD groups, do not entirely endorse the literature studies linking steatosis with chronic hepatic inflammation [35, 36]. Even spite the connection between lipid overload and the liver inflammatory response, demonstrating that FFAs can directly activate the IKK-β liver pathway through a cathepsin B-dependent lysosomal mechanism [37], this mechanism cannot explain the nuclear activation by NF-κB presented mainly by the SSD group. Indeed, given the number of activated nuclei by NF-κB presented by the SSD rats compared to physically active animals regardless of diet, our outcomes suggest that Pln can be, directly or indirectly, an independent activator of liver inflammatory paths once NF-κB is a key transcriptional regulator of inflammatory response [38, 39]. This increased activity of hepatic NF-κB in rats with NAFLD has been linked with enhanced liver expression of proinflammatory cytokines and Kupffer cells activation [40]. Moreover, the hepatic NF-κB inhibition attenuated the inflammatory response and Kupffer activation in fat diet experimental models [41], suggesting the pathogenic role of lipid overload on liver NF-κB-induced inflammation. The liver NF-κB nuclear overactivation found in physically inactive rats may also be related to the macrophage polarization pattern since NF-κB is directly linked to the control of genes responsible for different immune cells differentiation such as monocytes, i.e., the macrophage precursor immune cells [41]. Although not evaluated in our study, it is known that steatosis related to hepatic long-term lipid overload is strongly connected with an increased expression of inflammatory cytokines, such as IL-6 and TNF-α, by overloaded hepatocytes and also M1 macrophages [42-44]. This increased expression of inflammatory cytokines can further stimulate the T cells activation with more cytokines
production, promoting more differentiation of M1 macrophages and recruiting more lymphocytes in a signaling cascade that accentuates the inflammatory process [42-44]. This vicious cycle between the hepatic NF-κB overactivation and proinflammatory cytokines expression may be responsible for the inflammatory profile assumed by the resident macrophages, but also for the recruitment and infiltration of macrophages from other regions, resulting in the marked infiltration found in our results.

In addition to the NF-κB activation presented by physical inactive groups, the inflammatory reaction also appears to be closely associated with the number of nuclei showing DNA fragmentation. It is known that high concentrations of proinflammatory cytokines can induce apoptosis in hepatocytes under oxidative stress in animals submitted to high fat intake [45]. As showed by SSD and SFD rats, the hepatic macrophages proliferation with the expected production of proinflammatory cytokines can trigger inflammatory signaling pathways, mediating transcription factors involved in lipid metabolism and inducing hepatocyte apoptosis [46]. In addition to inflammatory apoptotic signaling induced by physical inactivity, fat overaccumulation in hepatocytes, as suggested by the exuberant steatosis-like vacuolization in SFD group, can synergistically result in hepatotoxicity triggering cell death, also called lipoapoptosis [47]. Among the possible mechanisms involved in hepatocyte lipoapoptosis, the caspase 8 activation and the inflammatory injury related to lipopolysaccharide-induced (LPS) inflammasome activation have been pointed out [48, 49]. Although these mechanisms linking apoptosis to lipid overload explain the results presented by the SFD group, the results showed by physical inactive group fed with standard diet might be explained by the intrinsic signalization of programmed death induced by the mitochondria activation pathway [50]. Other important mechanisms have also been advanced to explain the enhanced liver apoptosis in NAFLD, namely the JNK activation, oxidative and endoplasmic reticulum stress, microbiota influence, and particularly the activation of hepatic stellate cells (HSC) [51]. In fact, apoptotic hepatocytes can activate HSC in a vicious cycle that is also involved in hepatic fibrosis in patients with both NAFLD and NASH [52].

The large number of hepatocyte nuclei activated by NF-κB, apoptotic liver cells and the expressive presence of macrophages with proinflammatory phenotype may also explain the high collagenous tissue deposition exhibited by the FD-fed animals compared to SD-fed rats. High levels of nuclear activation by NF-κB and presence of M1 macrophage, as mainly presented by
SFD animals, are dominant clinical traits in the early stages of NAFLD but also in response to chronic hepatocellular stress [49] as imposed by chronic lipid overload. The presence of inflammatory and apoptotic cells as well, as found in SFD group, is directly linked to the activation of the major fibrogenesis factor responsible for liver extracellular matrix proteins deposition, the hepatic stellate cells (HSC) [53]. Based in our results, the lipid overload imposed by the chronic consumption of FD, possibly leads to fibrogenic deposition via immune system activation by HSC, given that HSC are highly responsive to liver microenvironmental disturbance and injury signaling, interacting with hepatic macrophages and lymphocytes triggering fibrogenic deposition [54, 55]. Moreover, despite not evaluated in our study, the HCSs are highly sensitive to proinflammatory cytokines being directly involved in the activation of inflammatory pathways, especially the NF-kB as expressed by FD-fed groups but especially in the SFD animals in a harmful vicious cycle [56]. Besides implicated in inflammation trigger, the activated NF-kB in HSC can promote the fibrogenesis increasing the HSC cells survival in an intricate signaling network between inflammation and liver fibrosis development [57]. Furthermore, since just physically inactive animals, but not the groups physically active, presented clinical signs such as high number of inflammatory cells, nuclear activation by NF-kB and apoptotic cells, our results suggest that the expressive hepatic deposition of collagenous tissue exhibited by SSD and SFD groups is perhaps mediated by inflammation. Moreover, given the significant higher fibrotic deposition presented by the physically inactive compared to active animals FD-fed, its clear that such parameters were aggravated by PIn suggesting a boosting effect of PIn. Indeed, once the parameters implicated in liver fibrosis were aggravated in the physically inactive animals FD-fed, our results suggest that PIn probably intensifies the hepatic fibrotic tissue expression modulating the inflammatory signals induced by the fat diet. Since the comparison between physically inactive animals showed that just the FD-fed rats presented significant fibrosis deposition, this also suggests that PIn might not be a determinant factor in liver fibrosis related to NAFLD induced by fat diet. Suggestion corroborated by the low fibrosis levels exhibited also by the AFD group.

The AFD group also presented a surprisingly and significantly higher level of voluntary physical activity (VPA) compared to the active group fed with a standard diet. The results might suggest a hypothetical mechanism of increased energy expenditure to avoid weight gain and obesity, justifying the higher levels of VPA and the absence of body weight differences between
AFD and ASD group. However, there is no evidence in the literature supporting this hypothetical and compensatory mechanisms of fat diet control on VPA levels and vice versa. In fact, given the rodents physically active nature whether in captivity or natural environment, such hypothetical mechanisms contradict the models of obesity and other metabolic pathologies induced by different dietary modulations as well as the existence of obese animals in wild. On the other hand, it is known that VPA can modify the mechanisms of food intake control and satiety such as alterations in gastrointestinal hormone response, gastric emptying, and in metabolism and endocrine response of muscle and adipose tissue [58]. Simultaneously, VPA also influences the central control of satiety and food intake via hedonistic, rewarding and dopaminergic pathways through hormonal modulation, especially leptin and ghrelin, hormones able to inhibiting and stimulating food intake [59-61]. However, although it is not possible to identify a causal relationship for the pronounced VPA levels in AFD, it is the physical activity that explains the significantly lower NAFLD severity in AFD than in SFD animals. Especially the presence of hepatic steatosis-like vacuoles, contradicting the current evidence which suggests that, despite the physical activity be a well-known factor in maintaining metabolic balance, there is no conclusive evidence about the effect of reducing sedentary behavior or increasing physical activity on the incidence or severity of NAFLD [62]. Indeed, new evidence has demonstrated favourable impact of physical exercise on NAFLD. Despite the small impact on hepatic insulin sensitivity, mechanisms such as the improvement of peripheral and systemic sensitivity to insulin and consequently reducing of hepatic de novo lipogenesis have been indicated as crucial exercise benefits on NAFLD [63], explaining the lower presence of hepatic stems suggestive of steatosis in SFD animals. Moreover, physical exercise also acts directly on the clearance of very low-density lipoproteins, reducing liver fat overload [64] and indicating that not all the mechanisms related to changes in the hepatic fat content are only associated with improvements in insulin sensitivity. Even so, it’s important to note that the evidence regarding the physical activity effect on liver fat is still ambiguous since the contribution of both physical activity or exercise seems only to be clinically relevant when associated with weight loss [62, 65]. Moreover, it is undeniable that high VPA levels, as presented by the AFD group, are crucial for both maintenance and weight loss and consequently for the management of NAFLD. Indeed, the VPA levels demonstrated by the AFD group corroborates with the results related to BW which might partially explain the
difference in the presence of hepatic vacuoles suggestive of steatosis and consequently in NAFLD severity compared to SFD group.

Conclusion

Our study demonstrates the combined harmful effects of fat diet and PIn on the histological liver profile and metabolic parameters, driving to MS and NAFLD development. The results also suggest that the fat diet was more determinant than PIn in inducing NAFLD and MS. However, although PIn by itself was not decisive in the NAFLD development, it had a potentiating impact in the clinical parameters from NAFLD induced by fat diet, modulating the hepatic inflammatory response.

Acknowledgments

The authors would like to thank Mrs. Celeste Resende for technical assistance in sample preparation for histological analysis. The authors also thank for the support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasil). This study was financed by the Fundação para a Ciência e Tecnologia (FCT/UID/DTP/00617/2019).

Conflict of Interest

The authors declare that they have no conflict of interest.
References


23. Ma, Z., et al., Beneficial effects of paoniflorin on non-alcoholic fatty liver disease induced by high-fat diet in rats. Scientific reports, 2017. 7: p. 44819.
43. Luo, W., et al., Effect of modulation of PPAR-γ activity on Kupffer cells M1/M2 polarization in the development of non-alcoholic fatty liver disease. Scientific reports, 2017. 7: p. 44612.
STUDY 3

Antonio Bovolini, Ana Filipa Silva, Maria Amparo Andrade, José Alberto Duarte (2019). Langerhans islets phenotype alterations induced by fat diet and physical activity levels in Wistar rats.

Submitted to The International Journal of Applied and Basic Nutritional Sciences, Nutrition.
Langerhans islets phenotype alterations induced by fat diet and physical activity levels in Wistar rats

Antonio Bovolini, MSc a,*, Ana Filipa Silva, MSc b, Maria Amparo Andrade, PhD c, José Alberto Duarte, MD, PhD a**

a CIAFEL - Laboratory of Biochemistry and Experimental Morphology, Sports Faculty, University of Porto; R. Dr. Plácido da Costa 91, 4200-450 Porto, Porto, Portugal.

b Departamento de Cirurgia e Fisiologia, Faculdade de Medicina, University of Porto; Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Porto, Portugal.

c Federal University of Pernambuco; Av. Prof. Moraes Rego, 1235 - University City, Recife - Pernambuco, 50670-901, Brazil.

* Authors to whom correspondence should be addressed: Tel.: +351 22 04 25 200; Fax: +351 225 500 687; e-mail: 201108996@fade.up.pt and jarduarte@fade.up.pt
Abstract

**Background/Aim:** Physical inactivity (Pln) and fat diet (FD) are closely linked to metabolic syndrome (MS) development, overloading the endocrine pancreas seeking energy homeostasis. However, the relative contribution of FD and Pln to the pancreatic overload is unknown. This study aimed to verify the isolated and conjugated influence of FD and Pln in the Langerhans islets (LI) structure and function related to overload in Wistar rats. **Methods:** male Wistar rats were divided into four groups (n=10/group): active groups, fed with fat (AFD) or standard (ASD) diet; and physically inactive groups, fed with fat (SFD) or standard (SSD) diet for 21 weeks. Glucose tolerance (GT) and insulin sensitivity (IS) were assessed before sacrifice. Retroperitoneal adipose tissue and pancreas were weighted (PW), and pancreas samples processed for histological analyses. **Results:** Only the FD-fed animals presented MS. Comparatively to standard diet, FD impaired GT and IS, decreased PW and enlarged LI dimensions, with LI cellular death, inflammatory response, and enhanced collagen content, which were attenuated in AFD. Independently of the diet, Pln groups presented the higher amount of LI connective tissue, but without influence on inflammatory reaction and cellular death. The GT impairment was more notorious in FD-fed groups, while the decreased IS were more pronounced in Pln groups. **Conclusion:** FD induced MS with detrimental impact on pancreas overload, inducing LI morphological and function maladaptations, which were attenuated in active animals. Physical activity was not able to prevent FD-induced MS. FD showed a negative influence on glucose tolerance, while Pln mainly affected insulin sensitivity.

**Keywords:** pancreas overload, insulin sensitivity, glucose tolerance, inflammation, histology, metabolic syndrome.
1. Introduction

Chronic diseases such as type 2 diabetes (T2D) and obesity emerged as the leading health concerns over the past century. Significant scientific and clinical evidence suggests the prolonged consumption of foods containing high quantities of fat and sugar as one primary obesity and T2D drivers. This dietary pattern called "western diet" is strongly linked with dyslipidemia, high blood glucose levels, and hyperinsulinemia, with detrimental effects on Langerhans islets mainly in β cells. Intimately linked to metabolic syndrome (MS) and obesity, physical inactivity (PIn) has also been undeniably linked to disorders in glucose homeostasis and lipid metabolism. Although its direct pathogenic influence is unknown, it is established that the lack of muscle contraction is related to energy unbalance, glucose uptake impairment and increased insulin circulating levels. The sustained increase in insulin demand may impose an islets overload leading to functional and morphological adaptations seeking to restore the energy balance. Recent evidence from western diet models has shown that the long-term consumption of western diet with elevated fat diet content induces morphological alterations in endocrine pancreas of rodents. Although this compensatory response is already studied and described in animal models of dietary manipulation, the influence of PIn on this pancreatic response is still unidentified despite the strong clinical and epidemiological evidences linking PIn with MS and T2D. Indeed, both PIn and FD seems to be directly or indirectly implicated in MS and T2D development, despite unknowing the relative contribution of each one for the underlying pancreatic overload. Consequently, this study aimed to verify the relative influence of FD and PIn, applied isolated or conjugated, in the Langerhans islets phenotype related to overload in Wistar rats. Given the stressful psychophysiological impact cause by forced exercise training protocols on rodents, a voluntary physical activity to mimic the spontaneous physical activity expressed by animals in nature was adopted in our study, using cages equipped with running wheel. In this model, control animals are those voluntary physically active receiving standard diet. Independent variables are fat diet and physical inactivity, which was imposed restricting the animal movement to their cage space, without access to a running wheel.
2. Material and Methods

2.1. Animals and experimental design

Male Wistar rats, 4 weeks old (183.55±3.48 g), were purchased from Charles-River (France) and kept in a room with controlled temperature (22ºC), relative humidity at 60±10% and inverted 12h light-dark cycle. Rodent maintenance and the experimental protocol followed the guidelines defined by the European Council Directive (2010/63/EU), transposed into Portuguese law (Decreto-Lei n.º 113/2013, de 7 de Agosto), have been approved by the local ethical committee. After 1-week acclimatization fed with a standard diet, the animals were randomly divided into 4 groups: animals physically inactive fat-diet fed (SFD, n=10), animals physically active fat-diet fed (AFD, n=10), animals physically inactive standard-diet fed (SSD, n=10) and physically active animals standard-diet fed (ASD, n=10). The experimental protocol had 21 weeks length.

2.2. Diet

The animals were fed with a standard diet (A04) or high-fat diet (HFD; diet purified HF 231) from Scientific Animal Food & Engineering (SAFE, France) for 21 weeks. Both water and food were provided ad libitum. The dietary composition was supplied by the manufacturer (SAFE, France) and it is described in Table 1.

**Table 1. Standard and high-fat diet composition**

<table>
<thead>
<tr>
<th></th>
<th>Fat</th>
<th>Protein</th>
<th>Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard diet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>3.1%</td>
<td>16.1%</td>
<td>60.4%</td>
</tr>
<tr>
<td>ED (Kcal)</td>
<td>8.4% (279)</td>
<td>19.3% (644)</td>
<td>72.4% (2416)</td>
</tr>
<tr>
<td><strong>High-fat diet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>40.2%</td>
<td>28.7%</td>
<td>14.2%</td>
</tr>
<tr>
<td>ED (Kcal)</td>
<td>67.8% (3618)</td>
<td>21.5% (1148)</td>
<td>10.6% (568)</td>
</tr>
</tbody>
</table>

Percentages of macronutrients composition (MC) and energy density (ED) from standard diet (3.339,00 Kcal/Kg) and high-fat diet (5.335,80 Kcal/Kg). Data regarding energy contribution and diet composition were provided by SAFE (France).
2.3. Voluntary physical activity (VPA) and imposed physical inactivity (PIn)

The animals from physically active groups (ASD, AFD) were individually housed in cages equipped with activity-wheel and distance counter. The active animals had free access to the activity-wheel and the running distance was weekly recorded, being the results presented as Km/week average. The animals from the physically inactive groups were individually housed in cages without activity-wheel restricting the physical activity to their cage space. Both cages had an identical floor area (800 cm²; Tecniplast, Buguggiate, Italy).

2.4. Metabolic and blood pressure evaluation

The metabolic tests, namely glucose tolerance, triglycerides (TGC), and total cholesterol (TC) were performed three days before the animals’ sacrifice using blood from the tail vein obtained with a 25-gauge needle. After 6h fasting, the TGC and TC were measured at baseline. For the glucose tolerance test (GTT), glycemia was measured at baseline, and 15, 30, 60, 90, and 120 min after administration of 1g/kg of glucose by gavage. Two days before the animals’ sacrifice and after a fasting period of 6h, the resting blood pressure and the insulin sensitivity were assessed. The blood pressure was determined using an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT) as described before 14,15. The animals were placed into an animal holder 15 min before the blood pressure assessment to prevent stress-related variations. Blood pressure (BP) was measured during 10-minute sessions until we obtained 3 stable recordings. Insulin sensitivity was tested (IST) according to the methodology described elsewhere through the measurement of glycemia at baseline, and 15, 30, 60, 90, and 120 min after 0.5 U/kg intraperitoneal insulin injection. Glycemia and lipid profile were measured using Cholestech LDX® Lipid Profile Glucose cassettes by the Cholestech LDX System (Cholestech Corporation, Hayward, CA) following the manufacturer’s instructions.

2.5. Metabolic syndrome assessment criteria

Following the World Health Organization definition for human criteria (WHO)16, animals were classified as having metabolic syndrome in the presence of impaired glucose tolerance or insulin sensitivity with the concomitant presence of two or more alterations, such as central obesity, elevated blood pressure, and dyslipidemia (high TGC or TC content). The existence of
any of these abnormalities in each animal was considered for values above the percentile 100 of the ASD (control group).

2.6. Animal sacrifice, samples collection, and histological preparation

After 21-weeks experimental period, the animals were weighed, anesthetized with an intraperitoneal injection of Ketamine (90 mg/kg, Merial, France) with Xylazine (10mg/kg, Bayer, German), and euthanized by exsanguination after 6 hours fasting. Pancreas and retroperitoneal adipose tissue (RAT, as a marker of central adiposity) were harvested, washed in PBS (pH 7.2), and weighed in a precision balance (resolution 0.01mg; Kern 870, Balingen, Germany). Relative pancreas weight (rPW) and relative RAT (rRAT) were calculated for each animal by the respective ratio for body weight and expressed as a percentage (%). The pancreatic samples were immediately processed for light microscopy. The pancreas samples were fixed 24h in 4% paraformaldehyde solution at 4ºC, dehydrated through graded ethanol solutions, cleared in xylene and mounted in paraffin. Sections of 5µm thickness were cut, and slides were stained with Hematoxylin-Eosin (H&E), Picrosirius Red, or prepared for TUNEL or immunohistochemistry analysis.

2.6.1. Langerhans islets cross-sectional area assessment

After routinely stained with H&E, the pancreas sections were photographed by an optical microscope (Axio Imager A1; Carl Zeiss, Oberkochen, Germany) coupled to a digital camera (LEICA DM4000B; Nussloch, Germany) and the Langerhans islets´ cross-sectional area (CSA, µm²) measured using the NIH ImageJ software (Image Processing and Analysis in Java, USA). To assess this CSA at least 30 of Langerhans islets (LI) were analyzed per animal.

2.6.2. Langerhans islets collagen tissue content assessment

Pancreas sections were stained with Picrosirius Red according to the method of Sweet et al. 17 by incubation on 0.1% Sirius red picric acid for 90 min. Them, sections were rinsed in 0.5% acetic acid, dehydrated in ethanol and cleared in xylene. The software Image-Pro Plus 6.0 (Media Cybernetics, Inc) was used to quantify the percentage of area (µm²) covered by the colors corresponding to the respective tissues. Respectively, the collagen and remained pancreatic
tissue stain in light red and yellow. To assess collagen tissue content at least 30 LI were analyzed per animal.

2.6.3. Langerhans islets cellular apoptosis assessment

Terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) technic was used to assay the presence of pancreas apoptotic nuclei with a commercial kit according to the manufacturer instructions (In situ cell death detection kit, Fluorescein (Cat. 11684795910, Roche Applied Scientific, Indianapolis)). After routine deparaffinization, sections were immersed in 0.1M citrate buffer (pH 6.0) and submitted to pressure cooker for 10 min. After cooled at room temperature immersed in citrate buffer, sections were rinsed in PBS and blocked with 3% bovine serum albumin solution for 30 min at room temperature and, then, incubated in freshly prepared TUNEL nucleotide mixture in a humidified and dark chamber at 37ºC for 60 min. Negative and positive controls were simultaneously prepared by incubation with label solution only (nucleotide mixture) or by incubation with deoxyribonuclease I (DNase I, cat. 10104159001, Sigma-Aldrich) before the labeling procedure, respectively. The number of positive nuclear staining was expressed as nuclei/µm². To assess TUNEL positive nuclei, at least 30 LI were analyzed per animal.

2.6.4. Langerhans islets NF-kB activation and macrophage phenotype assessment

Pancreas deparaffinized sections were rinsed in distilled water and incubated in PBS for 10 min previously the antigen retrieval in a pressure cooker for 10 min in 0.1M citrate buffer (pH 6.0). After cooled at room temperature immersed in citrate buffer, the sections were washed twice (5 min each) with PBS solution and the endogenous peroxidase activity was blocked in a fresh 3% solution of hydrogen peroxide in methanol for 30 min. The non-specific binding sites were also blocked with 3% BSA for 30 min. Following the blocking steps, each slide was incubated with anti-NF-kB p50 polyclonal rabbit antibody (diluted 1:50; sc114, Sta. Cruz), Anti-CD68 polyclonal rabbit antibody (diluted 1:100; ab125212, Abcam) for M1 macrophage quantification and anti-Mannose receptor rabbit polyclonal antibody (diluted 1:100; ab64693, Abcam) for M2 macrophage quantification in PBS-T overnight (4ºC).Slides were washed with PBS and then incubated with a goat anti-rabbit IgG peroxidase secondary antibody (diluted 1:150; Ab97051,
Abcam) in PBS-T for 2h at 37°C (the same secondary antibody was used for all labeling). The sections were then washed under gentle stirring and incubated with 3,3’-diaminobenzidine tetrahydrochloride (SIGMAFASTTM DAB, cat D4293-50SET, Sigma-Aldrich) reagent for 7 minutes. After the reaction interruption and subsequent washing, the sections were then counterstained with a solution of hematoxylin-water (dilution, 1:8) for 5 min. Negative controls were performed with the omission of the primary antibody incubation step. At least two sections/animal were evaluated per group for each evaluated structure. The number of NF-kB nuclear staining and macrophage-like cells in LI were expressed by the number of positive cells per µm².

2.7. Statistical analysis

The GraphPad Prism® (version 7.00, GraphPad Software, San Diego, California, USA) was used to do the statistical analysis. The Shapiro-Wilk test was performed to investigate the data normality. The obtained data were expressed as mean±standard deviation for normal distributed or as median with percentiles 25 and 75 (P25-P75) for abnormal distributed data. The one-way ANOVA followed by the Tukey post-hoc test was used to analyze data with normal distribution. The data with abnormal distribution (TGC, BP and CSA of LI) were analyzed with Kruskal-Wallis followed by the Dunn’s post-hoc test. The two-tailed unpaired T-test was used to analyse the physical activity differences among active groups. Differences were considered significant when p<0.05.

3. Results

3.1. Physical activity, metabolic and blood pressure assessment

The comparison of physical activity (PA) levels among ASD (8.932±0.588 Km/week) and AFD (19.201±1.169 Km/week) showed a surprising and significant increase (p<0.05) in PA performed per week by the group fed with fat diet. Results regarding TGC, TC, BP and central obesity evaluations are presented in Table . The FD-fed groups, AFD and SFD, exhibited a significant higher median and mean value of TGC and TC compared to SD-fed groups, SSD and ASD (p<0.05). The results also demonstrated the harmful fat diet influence on BP. The SFD group displayed significant increased BP compared to both groups fed with standard diet, SSD and ASD
(p<0.05), while the AFD just showed a significant increase of BP compared to ASD group (p<0.05).

**Table 2. Variables required for metabolic syndrome diagnosis.**

<table>
<thead>
<tr>
<th></th>
<th>TGC</th>
<th>TC</th>
<th>BP</th>
<th>rRAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SSD</strong></td>
<td>43.5 (40-69.75)</td>
<td>89.7±14.01</td>
<td>138 (134.8-141)</td>
<td>2.923±0.84</td>
</tr>
<tr>
<td><strong>SFD</strong></td>
<td>202.5 (76-256.80)</td>
<td>110.5±16.09</td>
<td>168 (148-183.5)</td>
<td>4.477±0.80</td>
</tr>
<tr>
<td><strong>ASD</strong></td>
<td>43 (37-61.75)</td>
<td>92.9±4.50</td>
<td>112 (99-116.5)</td>
<td>1.97±0.91</td>
</tr>
<tr>
<td><strong>AFD</strong></td>
<td>120 (86-186)</td>
<td>108.3±11.53</td>
<td>142 (118-145.3)</td>
<td>4.1±0.79</td>
</tr>
</tbody>
</table>

Triglycerides (TGC, mg/dl), total cholesterol (TC, mg/dl), blood pressure (BP, mmHg) levels, and relative retroperitoneal adipose tissue (rRAT, %) from physically inactive standard diet-fed (SSD), active standard diet-fed (ASD); physically inactive fat-diet fed (SFD) and active fat-diet fed (AFD) groups. The results regarding TC was expressed as means ± standard deviation while the outcomes related to TGC and BP were expressed as median (P25-P75).

π p<0.05 vs. SSD; 
µ p<0.05 vs. ASD;

Based on the P100 of ASD group (control group) for TGC, TC, BP, and rRAT, which were respectively 94 mg/dL, 100 mg/dL, 121 mmHg and 4.362%, the absolute frequency of animals from SSD, SFD and AFD groups classified as having dyslipidemia (high TGC or TC content), hypertension, or central obesity are described in Table 3.

**Table 3. Absolute frequency of animals in each group with values of triglycerides (TGC), total cholesterol (TC), blood pressure (BP), and central obesity (rRAT) higher than the percentile 100 of physically active standard diet-fed (ASD) control group.**

<table>
<thead>
<tr>
<th></th>
<th>TGC</th>
<th>TC</th>
<th>BP</th>
<th>rRAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASD</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>SSD</strong></td>
<td>2</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td><strong>SFD</strong></td>
<td>9</td>
<td>7</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td><strong>AFD</strong></td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

Legend: physically active fat diet-fed (AFD), physically inactive standard diet-fed (SSD), and physically inactive standard fat diet-fed (SFD).
### 3.2. Glucose tolerance test and insulin sensitivity test

The GTT and IST outcomes are depicted in Figure 1, being the respective area under curve (AUC) described in Table. Both FD-fed groups showed differences in AUC of GTT compared to SD-fed animals. Namely, the AFD group demonstrated significant increases (p<0.05) in AUC compared to both SSD and ASD groups while the SFD group shown an increased AUC (p<0.05) only compared to the ASD group. The AUC results from the IST showed that only the AFD group showed a significant increase (p<0.05) of AUC when compared to the control group (ASD). The remained groups did not show significant differences regarding AUC.

![Figure 1](image_url). Blood glucose concentrations (mg/dL) during the glucose tolerance test (GTT) and the insulin sensitivity test (IST) in physically inactive standard diet-fed (SSD, ○), physically inactive fat-diet fed (SFD, ●), active standard diet-fed (ASD, △), and active fat-diet fed (AFD, □).

| Table 4. Area under curve of glucose tolerance test and insulin sensitivity test. |
|-------------------|-------------------|-------------------|
|                   | GTT               | IST               |
| SSD               | 14,651±715        | 6,685±4147        |
| SFD               | 15,769±1184µ      | 7,044±393         |
| ASD               | 13,590±871        | 5,924±282         |
| AFD               | 16,420±721πµ      | 7,613±1539µ       |

Mean values ± standard deviation of area under curve (AUC, AU) relative to glucose tolerance test (GTT) and insulin sensitivity test (IST) from physically inactive standard diet-fed (SSD), physically inactive fat-diet fed (SFD), active standard diet-fed (ASD), and active fat-diet fed (AFD) groups. π p<0.05 vs. SSD; µ p<0.05 vs. ASD;
3.3. Metabolic syndrome classification

The P100 of AUC for GTT and IST registered in ASD were 14,313 AU and 6,278 AU, respectively. Based on the criteria defined to diagnose metabolic syndrome and on results of IST, GTT, BP, TC, TGC, and central adiposity assessed by rRAT, all animals from SFD (n=10) and AFD (n=10) filed the criteria to be classified with metabolic syndrome. In SSD group, any animal accomplishes the criteria for metabolic syndrome diagnosis.

3.4. Morphometric evaluation

Body weight (BW) as well as the absolute and relative weight of retroperitoneal adipose tissue and pancreas are depicted in Table. No differences in BW were registered among groups. Regarding pancreas weight (PW), the AFD group showed a significant decrease compared to both SSD (p<0.05) and ASD (p<0.05) groups. The SFD group also presented a decreased PW compared to the ASD group (p<0.05). When normalized by BW, the SFD and AFD groups displayed a significant reduction of rPW in comparison to ASD animals (both p<0.05).

Table 5. Body weight and weight (absolute and relative) of pancreas and retroperitoneal adipose tissue.

<table>
<thead>
<tr>
<th></th>
<th>BW</th>
<th>PW</th>
<th>rPW</th>
<th>RATW</th>
<th>rRATW</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSD</td>
<td>373.0±70.57</td>
<td>1.01±0.355</td>
<td>0.20±0.066</td>
<td>14.87±1.302</td>
<td>2.92±0.847</td>
</tr>
<tr>
<td>ASD</td>
<td>369.7±81.00</td>
<td>1.20±0.190</td>
<td>0.25±0.060</td>
<td>9.43±1.611</td>
<td>1.97±0.913</td>
</tr>
<tr>
<td>SFD</td>
<td>377.2±66.51</td>
<td>0.74±0.151</td>
<td>0.16±0.029</td>
<td>19.74±1.903</td>
<td>4.48±0.805</td>
</tr>
<tr>
<td>AFD</td>
<td>358.1±75.31</td>
<td>0.71±0.212</td>
<td>0.15±0.039</td>
<td>18.99±2.315</td>
<td>4.10±0.790</td>
</tr>
</tbody>
</table>

Body weight (BW, g), pancreatic weight (PW, g), relative pancreas weight (rPW, %), retroperitoneal adipose tissue weight (RATW, g), relative retroperitoneal adipose tissue weight (rRATW, %) from physically inactive standard diet-fed (SSD), active standard diet-fed (ASD); physically inactive fat-diet fed (SFD) and active fat-diet fed (AFD) groups. The results were expressed as means ± standard deviation. π p<0.05 vs. SSD; µ p<0.05 vs. ASD;
3.5. Langerhans cross-sectional area

The SFD group islets’ CSA median of 2908 (1721-5057) µm$^2$ was significantly higher (p<0.05) than SSD, ASD, and AFD groups, which CSA median values were 1743 (811.3-3045) µm$^2$, 1621 (894.5-2994) µm$^2$, and 1449 (772.3-2505) µm$^2$, respectively. The CSA results are depicted in Figure 2.

Figure 2. Representative light micrographs from pancreas of physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d) stained with hematoxylin-eosin. The yellow arrows identify Langerhans islets. Values of CSA are presented in e as a boxplot graphic.

π p<0.05 vs. SSD;
μ p<0.05 vs. ASD;
δ p<0.05 vs. AFD.

3.6. Langerhans islets collagen content

Compared to active animals, the sedentary groups presented a significantly higher collagen content in Langerhans islets (p<0.05) as depicted in Figure 3.

Figure 3. Representative light micrographs of pancreas stained with picrosirius red from physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d), depicting Langerhans islets. The red staining is indicative of collagen content. Mean values ± standard deviation of collagen content (%) in Langerhans islets of the different groups are presented in e.
μ p<0.05 vs. ASD;
δ p<0.05 vs. AFD.

3.7. Apoptosis assessment

Both groups AFD (9.35±1.755 cells/µm²) and SFD (10.75±2.828 cells/µm²) presented a higher number of Langerhans islets' apoptotic cells than SSD (7.65±2.207 cells/µm²) and ASD (5.95±2.212 cells/µm²) groups (Figure 4). No differences were found among groups fed with the same type of diet.

![Figure 4. Fluorescence micrographs of pancreas sections treated with terminal deoxynucleotidyl transferase dUTP method (TUNEL) representative of physically inactive standard diet-fed (SSD, a), physically inactive fat-diet fed (SFD, b), active standard diet-fed (ASD, c), and active fat-diet fed (AFD, d) groups, showing positive stained nuclei (red arrows) within the Langerhans islets. Mean values ± standard deviation of TUNEL positive nuclei/µm² are presented in e. π p<0.05 vs. SSD; μ p<0.05 vs. ASD.](image)

3.8. NF-kB activation and macrophage phenotype assessment

The number of positive nuclei stained for NF-kB (Figure 5) was significantly higher (p<0.05) in both fat diet-fed groups, AFD (21.7±6.76 cells/µm²) and SFD group (34.6±11.67 cells/µm²) when compared to both standard diet-fed groups, ASD (13.2±5.56 cells/µm²) and SSD (14.8±6.75 cells/µm²) groups. Within the fat diet-fed groups, SFD presented a higher positive cell number for NF-kB (p<0.05) comparatively to AFD. Regarding macrophage phenotype, both dietary manipulation and physical inactivity did not influence the number of M1 macrophages. Despite the significant difference (p<0.05) between SFD (1.00±0.743 cells/µm2) and ASD group (0.47±0.629 cells/µm²), no other differences were observed with SSD (1.03±0.964 cells/µm²) or
AFD (0.87±0.819 cells/µm²). For M2 phenotype no differences were observed among ASD (2.97±1.129 cells/µm²), AFD (2.63±1.098 cells/µm²), SSD (2.30±1.179 cells/µm²), and SFD (2.37±1.217 cells/µm²).

4. Discussion

The results demonstrate the determinant effect of fat diet intake on metabolic syndrome development independently of physical activity levels. Both FD-fed groups presented all the abnormalities necessary to fulfill the diagnostic for metabolic syndrome, and higher levels of physical activity were not able to prevent FD-induced MS. The abnormalities composing the MS were also accompanied by pancreatic morphological changes at the Langerhans islets with an increase in CSA, collagen content, inflammatory response, and cellular apoptosis.

Although the FD protagonism in the MS induction, the outcomes from GTT and IST indicate distinct roles of the FD and PIn on insulin sensitivity and glucose tolerance. While the FD was determinant to reduce glucose tolerance, the PIn played a more critical role in diminishing insulin sensitivity. It is known that physical activity (PA) levels induce adaptations in peripheral target organs, especially in skeletal muscle, liver and adipose tissue, enhancing insulin sensitivity and glucose uptake, even by insulin-independent pathways. In this sense, the more favorable IST results observed in the AFD group comparatively to SFD can be explained, among others, by
chronic muscular adaptations induced by regular PA such as increased capillary density, improvements on oxidative capacity and lipid metabolism, and raised insulin signaling proteins. The observed LI dimensions in AFD and SFD support this positive impact of PA, and the adverse effects of PIn on insulin sensitivity. Increases in LI dimensions has been described in animal models of high-fat diet and in obese patients, mainly explained as a compensatory mechanism seeking to increase insulin production and secretion resulting from hyperglycemia due to diminished insulin sensitivity in target organs. In humans, obesity has been associated with increases of approximately 50% in β-cell volume, being the hypertrophy more notorious than cell proliferation, consistent with the pronounced rise in RAT and LI dimension observed in the SFD group comparatively to AFD and the ASD control group. Although mechanisms are not fully understood, prolonged PIn favors a reduced insulin peripheral sensitivity, inducing islets hyperstimulation with compensatory morphological adaptations such as those presented by the SFD group. Indeed, the reduced glucose uptake and consequent increase insulin demand related to central obesity and PIn might explain the decreased insulin sensitivity and LI dimension presented by SFD group. In addition to reduced insulin sensitivity, PIn was also decisive in the pronounced fibrosis observed in both SSD and SFD groups comparatively to ASD and AFD groups. Studies in animal models and human patients have associated islet fibrosis with insulin resistance and reduced glucose tolerance, as presented by the SFD group. Moreover, the inflammatory response presented by SFD group adds up to the lipid overload as a fibrosis inducer in LI, since inflammatory signaling is fundamental for the development of fibrosis. It is also important to emphasize the pancreatic stellate cells (PSCs) activation have been pointed out as a critical factor in pancreatic fibrosis development, even not evaluated in our study. Indeed, recent studies also showed its presence of PSCs in the islets from diabetic rats, unveiling another important factor in the attempt to unravel the mechanisms involved in islets development of fibrosis. However, the pronounced LI fibrosis presented by the physically inactive groups, SSD and SFD, compared to the physically active groups, AFD and ASD, also demonstrated the predominance of PIn in the induction of islet fibrosis regardless of diet. On the other hand, the diet impact on glucose tolerance, as reflected by the GTT data, has probably a more complex mechanism involving the harmful influence of high levels of circulating free fatty acids (FFA) on multiple organs, with negative consequences on glucose homeostasis.
Indeed, elevated levels of FFA can trigger systemic abnormalities in glucose target organs especially the liver, skeletal muscle, adipose tissue, and pancreas leading to a diminished peripheral insulin sensitivity and ultimately to a reduced insulin secretion due to pancreas failure. The combined action of high levels of FFA and glucose in the pancreas induces β-cell apoptosis via glucolipotoxicity, supporting the hypothesis that the lipotoxicity only occur in the presence of high levels of glucose, and it is unlikely the former to happen in the absence of the second. Moreover, the uninterrupted and progressive increase in insulin demand can also induce β-cell apoptosis via oxidative stress, and chronic inflammation. Indeed, the expressive NF-κB nuclear activation presented the pancreatic islet from both groups FD-fed may also explain the elevated apoptotic cells, since NF-κB nuclear activation exert a mostly pro-apoptotic role in pancreatic cells, especially in β-cells. Evidence suggests that there is no detectable NF-κB activity in resting β-cells, but their exposure to a pro-inflammatory environment can activate and translocate the NF-κB to the nucleus by inducing the production of genes that regulate the dysfunction and cell death via apoptosis. The inhibition of cytokine-induced nuclear activation of pancreatic NF-κB prevented cytokine-induced cell death in both human and animal (rodents) pancreas, suggesting the crucial NF-κB function in pancreatic cell death by apoptosis and pancreas failure, and explaining the high number of apoptotic cells in LI and the elevated glucose levels. The increased islet population of M1 macrophages presented by the SFD and AFD groups also appears to be behind their marked nuclear activation of NF-κB, once activated macrophages secrete soluble mediators such as cytokines and oxygen free radicals that contribute to NF-κB translocation and nuclear activation.

5. Conclusion

Fat diet induced the development of metabolic syndrome with a detrimental effect on endocrine pancreas overload, causing morphological and function maladaptations in Langerhans islets. Physical activity had an attenuating impact; however, it was not able to prevent the metabolic syndrome development caused by fat diet. Also, the results reveal that fat diet showed a negative influence on glucose tolerance, while PIn mainly affected insulin sensitivity.
6. Conflict of interests
The authors declare no conflicts of interest.

7. Bibliography


8. Acknowledgments

The authors would like to thank Mrs. Celeste Resende for technical assistance in sample preparation for histological analysis. The authors also thank for the support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasil). This study was financed by the Fundação para a Ciência e Tecnologia (FCT/UID/DTP/00617/2019).
CHAPTER IV

GENERAL DISCUSSION
The present chapter is divided into two sections that respectively provide a discussion of the methodological approaches (1) and a general discussion of the results (2).

**DISCUSSION OF METHODOLOGY**

The experimental design characterized by the imposed physical inactivity and the voluntary physical activity was fundamentally motivated by the current literature methodological contradictions and the lack of knowledge related to PI
clinical implications [34-36]. Indeed, most of the evidence related to physical inactivity comes from human epidemiological studies or extrapolations of animal models of physical exercise. However, the methodological discrepancies among epidemiological studies generate inconclusive data or are unable to describe the isolated influence of physical inactivity. Indeed, the methodological differences regarding the definition used to characterize sedentary behavior, and the use of subjective tools to evaluate the physical activity levels (mostly questionnaires and interviews), generate inconclusive results. Meanwhile, most of the published studies using animal models adopt programs of forced exercise with short duration [37-39]. However, programs of forced exercise impose a significant and recognized psychophysiological stress on animals capable of promoting deleterious adaptations (25, 26). Moreover, such programs clearly do not mimic the usual routine of intermittent exercises from rodents in nature. Likewise, experimental studies with rodents usually adopt animals with physical activity restricted to the cage space as control animals opposing to the physically active
nature of the animals. Based on this, free access to a running wheel seems to be a more appropriate methodological approach allowing animals to run freely and intermittently. Since the objective of the work that supported this thesis was to investigate the effects of physical inactivity, the animals without access to the running wheel comprised the experimental group. At the same time, the animals with free access to the running wheel were adopted as a control group by mimicking the animals’ active nature in nature, certifying their character as control groups.

Contrary to the physical (in)activity protocol adoption, the chosen fat diet was mainly based on its well-known efficiency in inducing metabolic disorders associated with obesity, mimicking the so-called "western diet" pattern [40-42]. Food pattern currently globalized and continuously implicated in the origin of the epidemic NCDs such as T2D and MS [13, 43]. Another key point in designing animal studies with dietary manipulation is that fat-rich diets meet the animals’ minimum nutrient requirements, especially proteins, vitamins, and minerals, eliminating the possibility of excessive dietary intake to meet these nutritional needs [44]. Also, like the physical activity protocols, the dietary manipulation studies also have lack of outcomes resulting from long-term protocols. Indeed, the dietary manipulation protocols are usually focused on caloric restriction in fetal or early development stages, or in short periods of hypercaloric dietary supplementation [45-48].

Such methodological misconceptions do not allow to observe the phenotype resulting from the long-term exposition to harmful environmental factors, particularly those belonging to the lifestyle, continuously implicated in the human origin of NCDs and currently widespread in human society [9, 13, 49, 50].
To avoid such methodological misunderstandings, this study prioritized the use of a 21-week long experimental protocol seeking to reproduce the environmental conditions implicated in the origin of metabolic disorders in humans — namely the association between the chronic consumption of unbalanced diets and physical inactivity. To observe the phenotypic response, mainly the diet and PIn impact, to long-term exposure to such environmental factors, 4-week old animals were selected because it is at this stage that the pups increase in weight, size and begin to ingest solid foods after breastfeeding and also to be biologically immatures [51, 52].

To that end, to identify the hepatic and pancreatic response seems to be an efficient methodological approach to identify the influence of environmental factors on phenotypes capable of predisposing to the NCDs development. The investigation of both organs was based mainly on the high liver and pancreas responsiveness to alterations in the metabolic environment and its determinism the metabolism balance. Knowing the relationship between the unbalanced eating patterns, physical inactivity, and the metabolic disorders’ development [13], as well as the metabolic determinism exercised by both organs [33, 53], identifying structural and functional changes at the end protocol, was the most logical methodological path to be followed.

Likewise, to analyze the PIn and fat diet impact on phenotype determination should also go through the structural and functional assessment of visceral and subcutaneous adipose as well as skeletal muscle tissue. Both organs are fundamental in the metabolic balance similar to the liver and pancreatic tissue, mainly due to the endocrine action exerted by the skeletal
muscle and adipose tissue, regulating hormones production such as leptin, insulin, and ghrelin. [54-56].

However, despite being an initial goal, the histological and biochemical analyses of both tissues were infeasible due to tissue preservation problems and the technical specificity necessary to do it. In particular, cryogenic techniques that would allow not only the adipocyte vacuoles content preservation but also the content of vacuoles indicative of ectopic fat deposition in liver and skeletal muscle were not possible to execute. Moreover, issues regarding blood samples preservation also impose limitations to its use. Notably, the serum markers capable of characterizing the metabolic environment resulting from the long-term exposure to environmental factors since early ages, unraveling the crosstalk between fat diet and principally PIn in inducing MS. However, the methodological limitations regarding the use of blood samples, adipose, and skeletal muscle tissue, the objectives of this work were not compromised.

Moreover, despite the impossibility to analyze the steatosis-like vacuoles content, the NAFLD classification was not affected. The ‘NAFLD activity score’ is the most widely used histological classification and diagnosis system for NAFLD in clinical research [57]. Indeed, the liver biopsy is the conclusive examination for NAFLD and provides a reliable assessment of clinical liver signals, namely steatosis, hepatocellular injury, inflammation, and fibrosis.

The MS definition in rodents does not exist, and consequently, we had to adopt the human criteria for MS diagnosis in animals. Additionally, the cutoffs to define hyperglycemia, hypercholesteremia, insulin resistance, glucose tolerance, and hypertension were created based on our own criteria. Indeed, in order to
assure the MS diagnosis reliability, we adopted the control group 100th percentile (P100) as cut-off values for all components evaluated. The metabolic disorders presence was only diagnosed when the animals presented values above the P100 values for the respective components evaluated compared to the control group. Also trying to assure the reliability of MS diagnosis, it was decided to use the World Health Organization (WHO) definition to MS diagnosis [58] because it requires the presence of T2D, IR or impaired glucose tolerance plus to two or more risk factors, ensuring the protocol's efficiency in inducing metabolic homeostatic unbalance. Besides ensuring the protocol's reliability, such requirement indicates the WHO definition as a more appropriate research tool while the ATP III [59] and IDF [60] seem to be more useful for clinical practice since they only require the fasting blood glucose assessment, while the WHO definition may require an oral glucose tolerance test.
DISCUSSION OF RESULTS

The results presented in study 1 demonstrate the impact of long-term exposure to environmental lifestyle factors, the high-fat diet, and PIn, on the adult phenotype of Wistar rats. In contrast to the current literature, at the end of 21 weeks of a hypercaloric and hyperlipidic diet administration, no body weight gain was observed. Instead, the animals fed with the fat diet exhibited an unexpected BW decrease followed by an expressive (and contradictory) retroperitoneal fat deposition regardless of physical activity levels. However, despite the contrast with most published studies using animal models of hypercaloric dietary manipulation usually associated with overweight and obesity [61, 62], the results from the original studies 2 and 3 corroborate with the often suggested adipocitary mediation in MS development [63, 64]. Indeed, the last decades’ evidence has undeniably revealed the visceral adipose tissue as a highly active endocrine organ and as the preponderant interlocutor between lifestyle and the development of disorders and metabolic diseases [65, 66], as observed in our studies independently of weight gain. Moreover, since only the fat diet-fed groups fulfilled the diagnosis criteria for MS, the results clearly demonstrate the diet predominance over PIn in inducing the metabolic disorders belonging to the syndrome cluster of risk factors.

By itself, an individual's body weight is a gross indicator of central adiposity despite being associated with overweight and obesity [67, 68]. Moreover, despite being a common clinical tool, the BW is a poor clinical indicator of an individual's body composition. As observed in our results, the high fat-diet chronic
consumption induced a significant increase in central adiposity, namely in retroperitoneal fat tissue, without increasing the animals’ body weight and regardless of physical activity levels. Different from the found results, the weight loss, as well as the physically active lifestyles, has been implicated in the improvement of clinical health markers such as the components of lipid profile, insulin sensitivity, and glucose tolerance [69-72]. Moreover, it is also possible to observe that PIn per se was not able to induce metabolic disarrangements, suggesting that PIn does not appear to be an autonomous MS pathogenic factor, being, instead, a debilitating factor of metabolic flexibility. Indeed, the PIn booster effect on the metabolic disorders induced by the high-fat diet chronic consumption seems to be also explicit, since the deleterious effects presented by the original studies were more notorious in physically inactive animals. These results corroborate with the pathogenic association between PIn and unbalanced diets indicated as the central cause of metabolic diseases epidemic usually associated with weight gain and obesity [73, 74], although not observed in our study. It is also important to emphasize that, despite presenting significantly higher rates of voluntary physical activity than animals fed with the standard diet, higher levels of physically active were not capable of avoiding the metabolic disarrangements and, consequently, the metabolic syndrome development by the physically active group fed with fat diet. Surprisingly, these results contradict the beneficial and well-documented physical activity effects on health markers regardless of its intensity, especially on the metabolic syndrome parameters [75].

The original studies two and three reveal that higher levels of physical activity were also not able to avoid the high-fat diet noxious repercussions on the liver and endocrine pancreas. Interestingly, the studies demonstrate that the high-fat
diet and physical inactivity induced distinct liver and endocrine pancreas maladaptive responses followed by the homeostatic balance loss at the end of 21 weeks of the experimental protocol. Indeed, the results presented by the second original study show the high-fat diet predominance in inducing NAFLD development in fat diet-fed animals with an intense steatosis-like vacuolization, a high number of apoptotic hepatocytes, and a significant collagen content deposition independently of physical activity levels. However, it is necessary to emphasize that the high-fat diet was not decisive in the hepatic inflammatory response, a central etiological pillar from the syndrome-related NAFLD. The liver maladaptations to high-fat diet chronic consumption are consistent with those described in the literature, especially the NAFLD development [76, 77]. Contrary, the results presented by physically active animals fed with the high-fat diet differ from the evidence regarding the benefit of weight loss and physical activity in the ectopic accumulation of hepatic fat [78-81].

Moreover, unlike found in liver, the fatty diet was the determinant factor in the endocrine pancreas inflammatory response observed in the fat diet-fed animals independent of physical activity levels. Namely, the expressive NF-κB nuclear activation and islet population of M1 macrophages presented the pancreatic islet from both groups FD-fed. Since the NF-κB nuclear activation and also M1 macrophage exert a crucial pro-apoptotic role in pancreatic cells, especially in β-cells [82], the diet influence on pancreatic response was also preponderant in inducing islet cell apoptosis, probably by glucolipotoxicity mechanisms [83]. Beside the preponderance on the liver and hepatic damage cited before, the diet was also fundamental to the glucose tolerance decrease reflected by the GTT data exhibited by the original study 3. Indeed, the results seem to endorse
evidence suggesting that chronic disturbances in fat metabolism can trigger systemic abnormalities in glucose target organs especially the liver, skeletal muscle, adipose tissue, and pancreas leading to glucose intolerance and diminished peripheral insulin sensitivity [84-86].

The animals’ long-term exposition to PIn induced a notable hepatic inflammatory response. Namely, the third study showed the PIn predominance in inducing an inflammatory liver environment in sedentary animals independently of administered diet, with a high number of hepatocyte nuclei activated by NF-kB and a macrophage population pro-inflammatory phenotype (increased M1 and decrease M2 phenotype). Furthermore, despite the high-fat diet predominance in inducing cell apoptosis in the endocrine pancreas, the hepatic cell death seems to have been mostly induced by the PIn, and probably mediated by the inflammatory response. The significant number of hepatic apoptotic cells found in physically inactive fat diet-fed animals is consistent with the apoptotic impact of high-fat diets on rodent hepatocytes related to local inflammation [87, 88]. The PIn predominance over fatty diet became even more evident due to the high number of apoptotic hepatocytes found in the physically inactive animals fed with the standard diet. The PIn preponderance over fatty diet was also observed in inducing morphological adaptations (increased islets cross-sectional area), and collagen content deposition in the endocrine pancreas. Although increases in islets dimensions have been described in animal models of high-fat diet and obese humans [89, 90], there is still no evidence from the PIn influence on the Langerhans’ islets morphology. However, although not fully understood, have been suggested that increases in islets dimension is an adaptative response seeking to increase insulin production due to the hyperglycemia resulting from
diminished insulin sensitivity in target organs [89, 90]. The results are in consonance with the high-fat diet impact on the islets dimension cited before, but also bring some light to the PIn role on the endocrine pancreas response to long-term physical inactivity. The outcomes also demonstrate that the endocrine pancreas long-term exposition to both environmental factors related to lifestyle imposes hyperstimulation of Langerhans islets leading to compensatory morphological adaptations. In addition to the islets’ morphological changes, the sedentary groups also presented repercussions on insulin sensitivity regardless of administered diet, indicating that PIn plays a crucial role in metabolic homeostasis. Indeed, differently than observed in GTT, the results exhibited by the fourth original study demonstrated that the PIn played a more critical role in diminishing insulin sensitivity then diet.
Our results provide support for the following conclusion:

- The long-term exposure, since the early phases of development, to a high-fat diet and physical inactivity results in an adult phenotype characterized by central fat deposition, hepatic, and pancreatic maladaptations.
- The high-fat diet chronic consumption favors the development of metabolic syndrome regardless of physical activity levels and body weight gain.
- Higher levels of voluntary physical activity are not able to prevent metabolic syndrome development and the associated hepatic and pancreatic maladaptations.
- Physical inactivity *per se* does not seem to be able to induce metabolic syndrome but modulates its severity, influencing the central fat deposition, metabolic alterations, the hepatic and pancreatic maladaptations, and also the loss of energy homeostasis caused by the high-fat diet long-term intake.
- Regarding to the metabolic syndrome components, glucoce intolerance is predominantly influenced by the high-fat diet while the loss of insulin sensitivity is mainly conditionated by physical inactivity.
CHAPTER VI

BIBLIOGRAPHY
Bibliography


66. Janochova, K., M. Haluzik, and M. Buzga, *Visceral fat and insulin resistance—what we know?* Biomedical Papers of the Medical Faculty of Palacky University in Olomouc, 2019. 163(1).


88. Dai, Y.-J., et al., *Chronic inflammation is a key to inducing liver injury in blunt snout bream (Megalobrama amblycephala) fed with high-fat diet*. Developmental & Comparative Immunology, 2019.
