

DISSERTAÇÃO DE CANDIDATURA A GRAU DE DOUTOR APRESENTADA À
FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

PROGRAMA DOUTORAL EM METABOLISMO, CLÍNICA E EXPERIMENTAÇÃO

**Unhealthy eating habits at a young age: impact on the inflammatory
response, oxidative stress, and neuroendocrine function**

**Hábitos alimentares não saudáveis no jovem: impacto na resposta
inflamatória, stress oxidativo e função neuroendócrina**

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Os seguintes trabalhos fazem parte desta Tese:

- Original paper: Early unhealthy eating habits underlie morpho-functional changes in the liver and adipose tissue in male rats
- Original paper: Unhealthy diets induced distinct and regional effects on intestinal inflammatory signaling pathways and long-fasting metabolic dysfunction in rats

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I. List of abbreviations

ALP: alkaline phosphatase

ALT: alanine transaminase

AST: aspartate transaminase

BMI: Body mass index

COX-2: cyclooxygenase 2

CVD: Cardiovascular disease

eCBs: endocannabinoids

GM: gut microbiota

HDL-C: high-density lipoprotein cholesterol

HSD: High-sugar diet

ICAM-1: intercellular adhesion molecule 1

iNOS: inducible nitric oxide synthase

LDL-C: low-density lipoprotein cholesterol

LPS: lipopolysaccharides

MetS: Metabolic syndrome

NAFLD: nonalcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

NF- κ B: nuclear factor kappa B

NLRP3: NLR family pyrin domain containing 3

NLRs: NOD-like receptors

PUFAs: polyunsaturated fatty acids

TAG: Triacylglycerols

TIID: Type 2 diabetes

TLRs: Toll-like receptors

VCAM-1: vascular cell adhesion molecule-1

WD: western diet

II. Figures and Tables index

Figure 1 - Trend and estimation in childhood obesity, worldwide.

Figure 2 - Central obesity and insulin resistance play a key role in the pathogenesis of metabolic syndrome in childhood and adolescence

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Table 1 - Diagnostic criteria for metabolic syndrome according to different organizations

Table 2 - The International Diabetes Federation's definition of the at-risk group and the metabolic syndrome in children and adolescents

III. Resumo

A síndrome metabólica é um conjunto de diferentes fatores de risco de cariz metabólico, incluindo obesidade central, hiperglicemia, hipertrigliceridemia, hipertensão e baixos níveis de colesterol de alta densidade. A obesidade central está associada a fatores de risco metabólico, e por isso, considerada preponderante no surgimento da síndrome metabólica. Em Portugal, metade da população apresenta excesso de peso ou obesidade e um terço da população tem síndrome metabólica. Quanto à obesidade infantil, 11,9% das crianças apresenta obesidade e 29,7% excesso de peso. Em 2019, a *Childhood Obesity Surveillance Initiative* (COSI) revelou que 80% da população infantil portuguesa consumiu produtos alimentares processados e, 71,3% consumiu refrigerantes açucarados, com uma frequência de cerca de 3 vezes por semana. Sabendo que, os hábitos alimentares se estabelecem na primeira infância, estes dados epidemiológicos são particularmente alarmantes. O consumo precoce de alimentos ricos em gordura e açúcar é reconhecido como um dos principais fatores de risco no desenvolvimento de disfunção metabólica e distúrbios relacionados, incluindo diabetes tipo II e esteatohepatite não alcoólica (NASH). Uma vez que a doença metabólica nos jovens pode ter consequências nocivas na saúde futura, o estudo e compreensão dos mecanismos fisiopatológicos subjacentes ao comportamento alimentar nestas idades é um desafio premente. Porém, as implicações associadas a comportamentos alimentares não saudáveis, como a dieta ocidental e/ou consumo excessivo de bebidas açucaradas por crianças ainda não está totalmente elucidado.

Para se compreender melhor as consequências a longo prazo desencadeadas pelo consumo crónico desses padrões alimentares em idade juvenil, foram avaliados os efeitos de duas dietas não saudáveis, na morfologia e função de órgãos como o fígado, tecido adiposo e tubo digestivo, bem como em parâmetros antropométricos e bioquímicos. Pretendeu-se aferir a associação dos efeitos destas dietas com a síndrome metabólica e com danos em órgãos-alvo, avaliando as principais vias associadas à disfunção metabólica, particularmente, a inflamação e o stress oxidativo. Ratos Wistar macho (21-23 dias de idade) foram submetidos a três diferentes grupos de intervenção dietética. O grupo controlo, com acesso a dieta padrão, o grupo dieta rica em açúcar (HSD) com acesso a dieta padrão e solução de sacarose e, o grupo dieta de cafetaria (WD) que consumiu a dieta padrão suplementada com uma seleção de produtos alimentares processados e acesso a solução de sacarose. A composição da WD utilizada

apresentava alta densidade energética (dieta hipercalórica), contendo uma percentagem elevada de gordura (especificamente gordura saturada) e de hidratos de carbono simples (nomeadamente sacarose). Após as 14 semanas de intervenção dietética, os ratos foram sacrificados, procedendo-se à recolha de amostras de sangue e tecidos (fígado, intestino delgado e intestino grosso e de tecido adiposo visceral). Os resultados revelaram que os níveis séricos de glicose, creatinina, as transaminases da alanina e do aspartato, assim como o perfil lipídico estavam aumentados em ambos os grupos expostos a dietas não saudáveis (HSD e WD). Ambos os padrões alimentares se mostraram obesogénicos, induzindo um aumento significativo do tecido adiposo visceral. Foram igualmente observadas alterações histopatológicas no fígado, incluindo organização anormal dos hepatócitos, acumulação de gotículas lipídicas e aumento da área fibrótica. Foram também observadas alterações na estrutura do tecido adiposo. Em ambos os grupos de dieta não saudável, a expressão hepática do Toll-like receptor (TLR)4, cicloxigenase 2 (COX-2) e E-Selectina encontrava-se aumentada, bem como os valores de peroxidação lipídica. No intestino delgado e intestino grosso do grupo WD, ocorreu um aumento significativo na expressão do gene do fator de transcrição nuclear kappa B (NF- κ B), dos recetores *TLR2* e *TLR4*, bem como da enzima sintase do óxido nítrico indutível (iNOS). Por outro lado, não se observaram diferenças na expressão de recetores do tipo NOD (NLR) contendo um domínio pirina 3 (NLRP3). A ingestão de bebida açucarada induziu uma resposta inflamatória, quantificável apenas no intestino delgado, através do aumento da expressão do *NF- κ B* e do *TLR2*, sugerindo o comprometimento da estrutura e função da barreira intestinal.

No geral, este estudo revelou efeitos negativos duradouros resultantes do consumo excessivo de sacarose (HSD) e de dietas ricas em gordura saturada e açúcar (WD). Na WD observou-se adiposidade mais pronunciada acompanhada de um fenótipo hepático caracterizado por aumento dos parâmetros inflamatórios e oxidativos, inflamação intestinal e por uma resposta inflamatória colónica exacerbada. Por outro lado, na dieta HSD observou-se uma resposta inflamatória no intestino delgado com impacto na estrutura e função da barreira intestinal. O índice de adiposidade aumentou com o consumo de ambas as dietas não saudáveis, embora não se tenham verificado alterações no ganho de peso corporal total.

IV. Abstract

Metabolic syndrome is characterized by a set of metabolic risk factors, including central obesity, hyperglycemia, hypertriglyceridemia, hypertension, and low levels of high-density lipoprotein cholesterol. Central obesity is strongly associated with metabolic risk factors; therefore, it is preponderant in the emergence of metabolic syndrome. Half of the Portuguese population is obese and has metabolic disorders. As for childhood obesity, 11.9% of children are obese and 29.7% are overweight. In 2019, the Childhood Obesity Surveillance Initiative (COSI) revealed that 80% of Portuguese children consumed processed food products and 71.3% consumed sugary soft drinks, with a frequency of about 3 times a week. These epidemiological data are particularly alarming because eating habits are established in early childhood. Consumption of foods high in fat and sugar at an early age is recognized as one of the main risk factors in the development of metabolic dysfunction and related disorders, including type II diabetes and non-alcoholic steatohepatitis (NASH). Since metabolic disease diagnosed in childhood and youth has deleterious consequences on future health, a better understanding of the pathophysiological mechanisms underlying unhealthy eating behavior is a pressing challenge. However, the implications associated with unhealthy eating behaviors such as the western diet and/or excessive consumption of sugary drinks by children are not yet fully clarified.

To understand the long-term consequences triggered by the chronic consumption of these dietary patterns at a young age, the effects of two unhealthy diets on the morphology and function of the liver, adipose tissue, and the digestive tract, as well as on anthropometric and biochemical parameters were evaluated. The association of these diets with metabolic syndrome and with the damage of target organs was assessed by evaluating the pathways strongly associated with metabolic dysfunction, particularly the inflammatory pathways and oxidative stress. Male Wistar rats (21-23 days of age) underwent three different groups of dietary interventions. The control group, with access to the standard diet, the high sugar diet (HSD) group with access to the standard diet plus sucrose solution, and the western diet (WD) group with access to the standard diet supplemented with a selection of processed food products plus sucrose solution. The WD composition used had a high energy density

(hypercaloric diet), containing a high percentage of fat (specifically saturated fat) and simple carbohydrates (namely sucrose). After 14 weeks of dietary intervention, the rats were sacrificed, and samples of blood, organs (liver, small intestine, and large intestine), and visceral adipose tissue were collected.

The results revealed that the serum levels of glucose, creatinine, alanine, and aspartate transaminases, as well as the lipid profile, were increased in both groups exposed to an unhealthy diet (HSD and WD). Both dietary patterns proved to be obesogenic, inducing a significant visceral adipose tissue weight gain. Histopathological changes were observed in the liver, including abnormal organization of hepatocytes and an accumulation of lipid droplets, by an increase in the fibrotic area. Changes were also observed in the structure of adipose tissue, specifically in adipocytes. In both unhealthy diet groups, hepatic expression of Toll-like receptor 4 (TLR4), cyclooxygenase 2 (COX-2), and E-Selectin was increased, as well as lipid peroxidation values. In the small intestine and large intestine of the WD group, there was a significant variation in the gene expression of the nuclear transcription factor kappa B (NF- κ B), the TLR2 and TLR4 receptors, as well as the inducible nitric oxide synthase enzyme (iNOS). However, there was no difference in the expression of NOD-like receptors (NLR) containing a pyrin 3 domain (NLRP3). The ingestion of a sugary drink induced an inflammatory response, quantified only in the small intestine, through increased expression of NF- κ B and TLR2, suggesting impairment of the intestinal barrier structure and function.

The study exposed the lasting negative effects of excessive sucrose (HSD) consumption and diets high in saturated fat and sugar (WD) in adipose tissue and systemic metabolic parameters. In WD, more pronounced adiposity and a hepatic phenotype characterized by increased inflammation and oxidative stress, intestinal inflammation, with an exacerbated colonic inflammatory response were observed. On the other hand, in the HSD diet, an inflammatory response was observed in the small intestine, disrupting intestinal barrier structure and function and causing metabolic dysfunction. The adiposity index increased with the consumption of unhealthy diets, although there were no changes in total body weight gain.

1. Introduction

1.1 Metabolic Syndrome

1.1.1. Definition

The metabolic syndrome (MetS) has had increased attention and research in the last decades, but in the medical literature, syndromes overlapping MetS have been described for nearly a century, always pointing towards a similar dysmetabolic phenotype. The term MetS was made official in a World Health Organization consultation panel in 1998 (Alberti & Zimmet, 1998). MetS encapsulates a combination of metabolic disorders, namely insulin resistance, glucose intolerance, elevated triacylglycerols (TAG), reduced high-density lipoprotein cholesterol (HDL-C), central obesity, and hypertension (Alberti et al., 2009; Catrysse & van Loo, 2017). MetS is therefore a clustering of individual metabolic risk factors. Obesity as is widely known is a significant threat to people's health, but the distribution of body fat has been under close investigation, as it is a determinant aspect to consider when assessing MetS risk (Bhaskaran et al., 2014). Central obesity was recognized as an independent risk factor for cardiometabolic diseases, a predictor of cardiovascular events (Bastien et al., 2014), and responsible for approximately 20% of all types of cancer, although, if combined with diet, for 35% (Wolin et al., 2010). Moreover, recent evidence gives support to the link between MetS and mental health conditions, as well as an increased risk of neurodegenerative disorders, including Alzheimer's disease (Arnold et al., 2018; Sarma et al., 2021).

In western society, MetS are becoming prevalent due to sedentary lifestyles and dietary patterns. A person is considered overweight when the BMI is between 25 and 30 and obese when the body mass index (BMI) is over 30. Currently, more than 1.9 billion people worldwide are obese or overweight, both are important risk factors for type 2 diabetes (T2D), cardiovascular diseases (CVD), and liver disturbances (Swinburn et al., 2011). Chronic liver disease associated with MetS ranges from simple steatosis to nonalcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis (Swinburn et

al., 2011; Samson & Garber, 2014). It is, however, of note that about 20% of NAFLD patients don't have MetS and, on the other hand, 10% with MetS don't have NAFLD (Samson & Garber, 2014). Furthermore, due to the impact on oxidative stress, inflammation, and metabolic homeostasis, MetS hamper a plethora of pathways that can lead to endocrine and vascular alterations (Rojas-Gutierrez et al., 2017) and, also, neuronal damage (Luque-Contreras et al., 2014; Minter et al., 2016).

Overnutrition, characterized by excessive intake of carbohydrates and fats, is one of the main factors contributing to an astonishing increase in obesity, particularly among minorities and socioeconomically disadvantaged populations. This excess caloric intake can trigger MetS, the cluster of metabolic abnormalities. The reported prevalence of the MetS greatly depends on the definition used, regarding several factors such as gender, age, socioeconomic status, and the ethnic background of study cohorts (Su et al., 2020). The definition of MetS is being harmonized but there are always additional iterations and discussions regarding MetS, its implications, and prevention. Waist circumference and CVD risk need to be further investigated, bearing in mind the different populations. The pathophysiology and the gene interactions sustaining the plethora of MetS components also need elucidation. MetS diagnosis would benefit from phenotypic pattern recognition, allowing clinicians to explore the presence of MetS components, which could give a closer estimate of the real risk of MetS.

1.1.2 Epidemiology

Obesity remains a major public health challenge in the 21st century (Fig. 1). According to the World Health Organization (WHO), the prevalence of obesity has nearly tripled since 1975. In 2016, almost 40% of adults were overweight and 13% were obese (Drozd et al., 2021). In the pediatric population, obesity has taken on epidemic proportions also. In children and adolescents aged 5 to 19 years, the prevalence of overweight increased from 4% in 1975 to more than 18% in 2016, and obesity from 1% in 1975 to 6% in girls and 8% in boys (Drozd et al., 2021). The Childhood Obesity Surveillance Initiative (COSI) carried out between 2015–2017 reports that childhood overweight and obesity have increased in recent decades, with

an estimated 124 million children and adolescents being obese and 213 million overweight (Spinelli et al., 2021). The rise in child obesity is also reaching worrying levels in Portugal. In the Child Nutritional Surveillance System – COSI Portugal, data from 2019 show that 11.9% of the children were classified as having obesity and, 29.7% as having overweight and obesity. Notwithstanding the worrying results, there was a reduction of 8.2 % of overweight in children, from 37.9% to 29.7%, and a reduction of 3.4% in childhood obesity, from 15.3% to 11.9%. For so, Portugal moved from the 2nd to the 14th place of the European countries with the highest prevalence of overweight children (INSA: Obesidade Infantil - INSA, 2022).

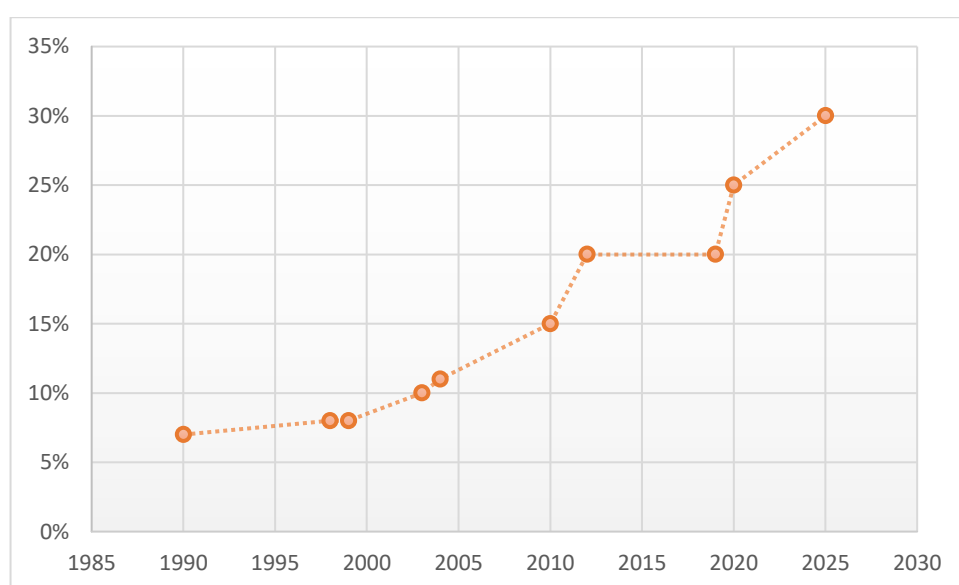


Figure 1 - Trend and estimation in childhood obesity worldwide. The increase in the consumption of high-sugar/high-fat food results in an increase in childhood obesity. The Atlas of Childhood Obesity of the World Obesity Federation estimates that by the year 2030 there will be 250 million obese children and adolescents. Adapted from Journal of Childhood Obesity ISSN 2572-5394 Vol.4 No.3, Nilly Shams

The prevalence of central obesity is high in Europe, the USA, and South Africa, in contrast to Asia (Reisinger et al., 2021). In European Region, overweight and obesity are more common in southern Europe. The reasons gathered to justify the higher prevalence in southern Europe are related to a lower stature for age, with higher birth weight, less breastfeeding time, less healthy eating habits, less physical activity, and parents' opinions on the real body weight status of their children (Spinelli et al., 2021). Furthermore, parental

history of obesity is closely associated with the presence of MetS in children and adolescents (Monzani et al., 2014).

Obesity and overweight during early childhood affect child development and it has been associated with an increase in chronic diseases in adulthood (Han et al., 2010; Styne et al., 2017). Children's and adolescents' weight is a consequence of environmental as well as genetic factors (Bel-Serrat et al., 2019; Styne et al., 2017). Children living in low- and middle-income countries are most at risk of having inadequate nutrition during the prenatal period (*The Double Burden of Malnutrition*, 2022). These children are also more exposed to nutritionally poor foods, which are rich in fat, sugar, and salt, as they are usually cheaper (*The Double Burden of Malnutrition*, 2022). In addition, overweight children and adolescents have a higher prevalence of MetS, with a variation from 10% to 57.4% among obese children and adolescents (Reisinger et al., 2021). Identifying children and adolescents with MetS is difficult due to the lack of clear recommendations on how to diagnose (Mameli et al., 2017). Body weight studies in children generally use the WHO definition or other proposed by the International Obesity Task Force (IOTF); however, these definitions of BMI categories differ, which causes some difficulty in comparing results from different studies. Nevertheless, prevention is the most viable action to contain the childhood obesity epidemic, whereas surveillance data is important for the effective analysis, implementation, and evaluation of health strategies and policies (Spinelli et al., 2021).

1.1.3. Pathophysiology

Excessive calorie intake and a sedentary lifestyle result in excess energy balance, which will be stored as fat. Visceral fat, for instance, is associated with an increase in insulin resistance, LDL-C, HDL-C, and very-low-density lipoprotein cholesterol (VLDL-C) (Fox et al., 2007) (Fig. 2). The pathophysiology underlying the metabolic changes induced by unhealthy diets is complex and involves several interrelated mechanisms. Experimental, epidemiological, and clinical evidence reveals that inflammation is not only implicated in the development of metabolic diseases but also in the complications that emerge from these pathologies (Hotamisligil, 2006).

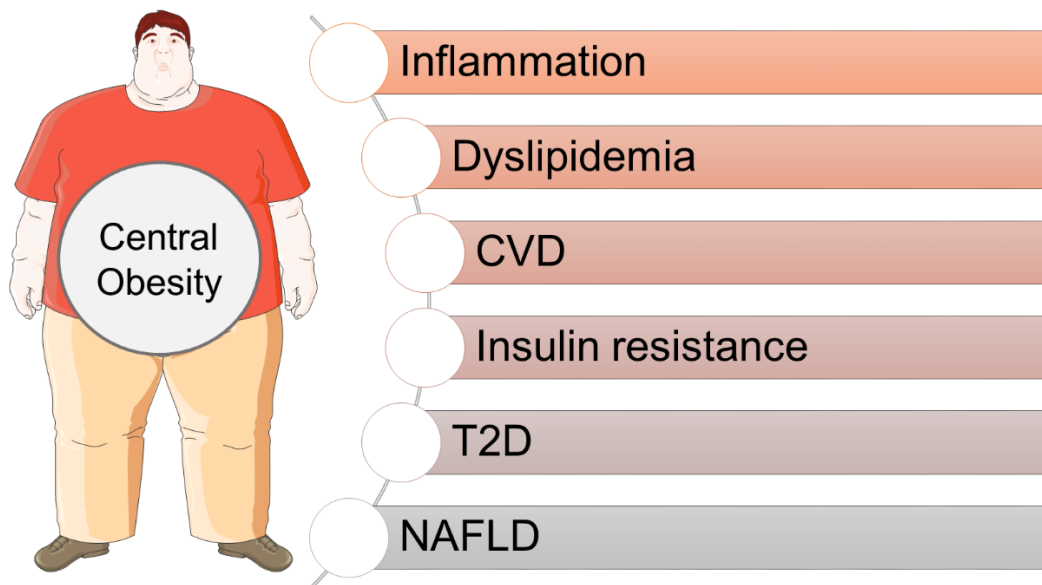


Figure 2 - Central obesity and insulin resistance play a key role in the pathogenesis of metabolic syndrome in childhood and adolescence. Central obesity is linked to several metabolic abnormalities like inflammation, dyslipidemia, type 2 diabetes (T2D), cardiovascular disease (CVD), and non-alcoholic fatty liver disease (NAFLD), particularly in children and adolescents. Central obesity and insulin resistance are major factors contributing to metabolic syndrome. Adapted from Helen H. Wang, 2020

Overfeeding activates several pro-inflammatory signaling pathways, leading to a condition of chronic low-grade inflammation in various metabolic tissues, affecting their normal function (Catrysse & van Loo, 2017). For example, in the adipose tissue, chronic low-grade inflammation resulting from excess saturated fatty acids in the diet is accompanied by inflammatory mediators and cytokines production (Wellen & Hotamisligil, 2005). Excess nutrient availability causes hypertrophic adipocytes leading to cellular degeneration and activation of inflammatory pathways through Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Abreu, 2010; Anitha et al., 2016; Burgueño & Abreu, 2020). Both the TLRs and NOD pathways converge to activate nuclear factor-kappaB (NF- κ B)-dependent gene transcription that regulates immune function and expression of genes associated with inflammatory mediators (Hörmann et al., 2014; T. Liu et al., 2017b; Malesza et al., 2021a; N. Wang et al., 2013). When these regulatory pathways fail, the development of metabolic dysfunction related to obesity and diabetes may occur (Nobs et al., 2020). The TLR-4, specifically, plays a critical role in glucose and lipid metabolism (Porrás et al., 2017a). Indeed, TLR-4 recognizes the saturated fatty acids in adipocytes and macrophages, which in turn leads to a reduction in insulin sensitivity and, ultimately, to insulin resistance (Könner & Brüning,

2012a). Cyclooxygenase 2 (COX-2) has a pivotal role in inflammation and its resolution phase. The role of the prostanoids' synthesis in the progression of the constellation of diseases related to MetS such as obesity, fatty liver, and diabetes is not completely clarified, but it is known that there is a rapid induction of COX-2 by peroxides, oxidative stress, NF- κ B cytokines, and chemokines (Hardwick et al., 2013). The eicosanoids derived from COX-2 activity take part in the inflammatory response, rising the synthesis and secretion of inflammatory mediators by both adipocytes and hepatocytes, leading to macrophage infiltration (Hardwick et al., 2013; Rogero & Calder, 2018).

In an obese state, macrophages change their number and location (Haase et al., 2014; Lumeng et al., 2007). Furthermore, adipocyte apoptosis contributes to macrophage infiltration into adipose tissue and drives macrophage polarization toward an inflammatory M1-like phenotype with increased phagocytic activity (Lindhorst et al., 2021). Macrophages, in the adipose tissue of obese individuals, are probably responsible for the increase in circulating inflammatory cytokines such as interleukin (IL)-6, IL-1 β and tumor necrosis factor α (TNF- α), contributing to an increased risk of CVD development (A. Gupta & Gupta, 2010). While in the liver and muscle there is a discrete inflammatory response, in white adipose tissue, there is a more pronounced systemic inflammatory response (Odegaard & Chawla, 2013). In obesity, this persistent pro-inflammatory state in which hypertrophic adipocytes and adipose tissue lead to increased pro-inflammatory cytokine levels contributes to insulin resistance and T1D (Ouchi et al., 2011). Therefore, a better understanding of the inflammatory signaling pathways in obesity can be a strategic avenue for the development of targeted therapeutic approaches for the metabolic complications that arise, allowing overcoming the limitations of traditional anthropometric obesity indices in the assessment of metabolic risk factors.

The finding that chronic low-grade inflammation underlies obesity changed the view about the determinant causes of obesity progression into MetS. It is now well accepted that an inflammatory process is activated early in adipocyte enlargement and adipose expansion and, during persistent obesity, shifts the immune system response towards a pro-inflammatory phenotype. Together these mechanisms have been identified as a causal link between obesity and inflammation (Lumeng et al., 2007). Of note, in this condition, are the appearance of inflammation in the pancreatic cells, brain, liver, intestine, and muscle, and the

interrelationship between inflammation, hepatic fibrosis, and angiogenesis (Saltiel & Olefsky, 2017). Among the chronic metabolic disorders, NAFLD is the most common chronic liver disease, which has been associated with a higher predisposition to worsen insulin resistance that leads to TIID (Ye et al., 2019). It can go from an increase in intrahepatic lipid content to the presence of inflammation, fibrosis, and cirrhosis (Cotter & Rinella, 2020a; Peng et al., 2021a). The NAFLD development has been explained by the theory of the double-hit hypothesis, considering insulin resistance as a primary insult that contributes to steatosis, triggering hepatic *de novo* lipogenesis (Til, & Moschen 2010). The second hit includes endoplasmic reticulum stress, mitochondrial dysfunction, hepatocellular apoptosis, as well as an increase in inflammatory responses (Til, & Moschen 2010).

MetS can be a predisposition towards metabolic deterioration in response to obesity development, caused by various factors including genetic, epigenetic, dietary, and environmental triggers (Carson & Lawson, 2018) (Fig. 3). Genome studies provided a useful tool for identifying certain gene loci linked to MetS predisposition (Povel et al., 2011). There are however several candidate genes, and the number of variants has been increasing in recent years (Povel et al., 2011; Wan et al., 2021). Although the underlying genetic architecture of MetS is under discussion, there is however evidence that the specific combination of certain traits may be important and may explain the large number of variants associated with MetS (Wan et al., 2021). In addition, there are several loci related to obesity and other MetS conditions, such as high TAG, high fasting insulin, and low HDL-C (Wan et al., 2021). For example, when pancreatic β cells are unable to compensate for insulin resistance, there may be a trait, and the genes associated with the prediction risk for TIID are linked with β -cell function, and not with a predisposition for obesity (Samson & Garber, 2014). However, only 10% of the multitude of MetS traits have been linked with specific genes (Povel et al., 2011; Samson & Garber, 2014).

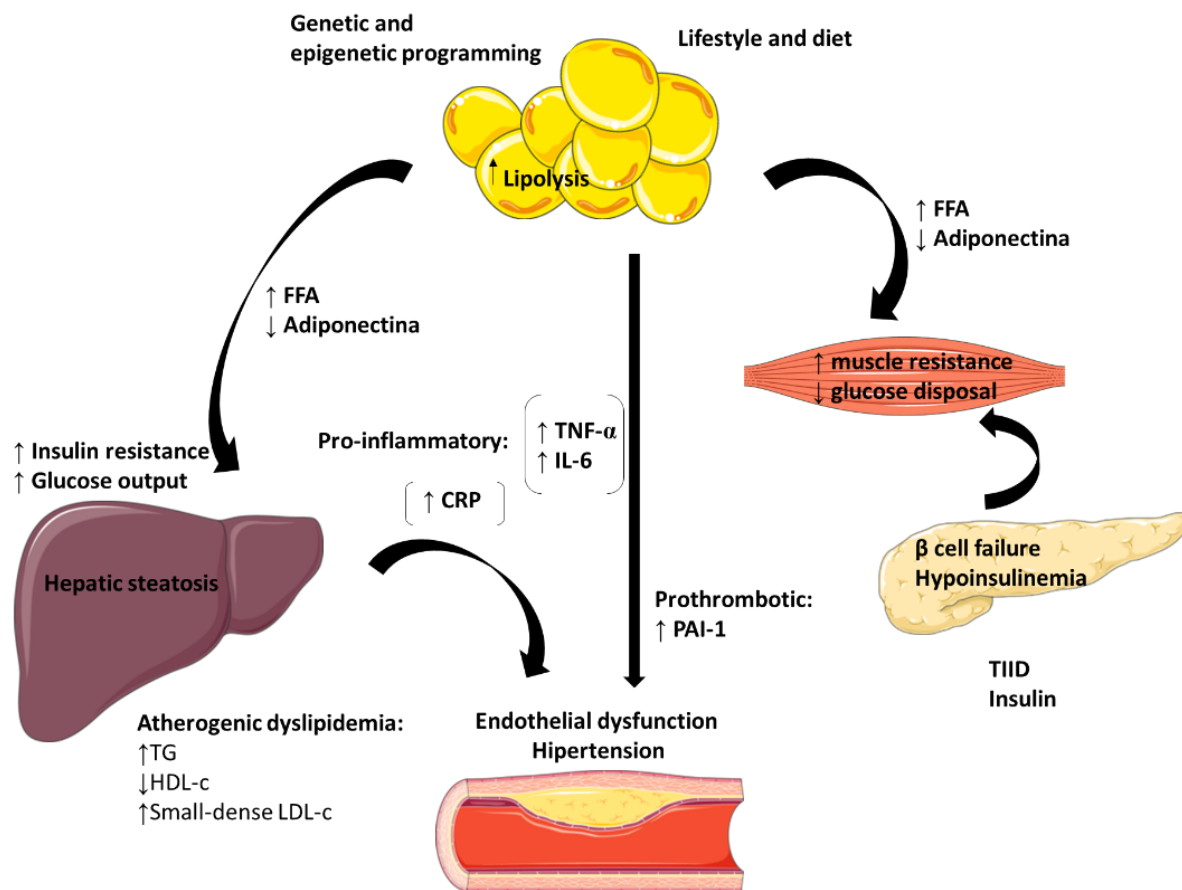


Figure 3 – A suggested mechanism for the clustering of metabolic syndrome (MetS) traits and the increased risk of T1DM and CVD. FFAs from visceral fat enter the portal circulation to be stored as TAG in the liver, stimulating the hepatic production of very low-density lipoprotein, and causing hypertriglyceridemia. Excess TAG is also transferred to LDL-C, a more interesting substrate for hepatic lipase, resulting in smaller, more atherogenic, dense LDL-C particles. FFAs migrate to peripheral tissues, potentiating the inhibition of insulin signaling. Excess FFAs and insulin resistance result in increased gluconeogenesis and hyperglycemia. Pancreatic β cells decompensate for the increased need for insulin to overcome insulin resistance, which can cause T1DM. The onset of hypertension depends on several factors, such as endothelial dysfunction because of OS as measured by FFAs, activation of the sympathetic nervous system because of hyperinsulinemia, inhibition of nitric oxide synthase, and the effects of adipose tissue-derived cytokines. The renin-angiotensin-aldosterone system is hyperactive in obesity. CRP: C-reactive protein, FFA: Free fatty acids, IL-6: interleukin 6, PAI-1: plasminogen activator inhibitor 1, TNF- α : tumor necrosis factor α . Adapted from Susan L. Samson, 2014.

During obesity, metabolic inflammation, characterized by the activation of pro-inflammatory signaling pathways and cytokine production in metabolic tissues, arises as a response to energy homeostasis impairment (Smith et al., 2018). An excess in fatty acid intake causes several organs to reach storage capacity, resulting in the infiltration of immune cells, and triggering an inflammatory process with a consequent increase of circulating inflammatory cytokines (Smith et al., 2018). The adaptive inflammatory response may also involve angiogenesis stimulation to counteract hypoxia and promote insulin resistance to

protect the fat cell from lipid overaccumulation. The overall degree of inflammation correlates with the severity of steady-state insulin resistance and plays a role in the adaptive response to overnutrition (M.-J. Lee et al., 2010; Shi et al., 2006). However, over time, the adaptive response converts into a persistent response, and the nature of chronic inflammation reveals a failure in the process of trying to resolve the initial insult (Wang et al., 2015). As consequence, various organs are affected by chronic inflammation (Lin et al., 2016; Weisberg et al., 2003).

Overnutrition can promote the expansion of adipose tissue, avoiding the ectopic deposition of lipids in other tissues, such as the liver and muscle, where it has lipotoxic effects (Sun et al., 2011). A healthy expansion of adipose tissue implies adipogenesis and angiogenesis to make room for new adipocytes and to meet their nutritional needs (Sun et al., 2011). Alongside adipocyte hypertrophy, the Western diet (WD) also causes other pathological alterations of adipocyte development, namely an abnormal architecture (Sun et al., 2011). If the expansion of adipocytes is not accompanied by a flexible extracellular matrix, as well as appropriate angiogenesis, normal cytoarchitecture can be altered (Johnson et al., 2016; Lee et al., 2010). A dysfunctional endothelium, characterized by an increased expression of cell adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1), is also involved in the gathering and joining of inflammatory cells to vascular endothelium (Antwi-Boasiako et al., 2018). In the early stages of the MetS, the recruited immune cells cross the endothelium to penetrate adipose tissue, and therefore, these initial inflammatory processes play an important role to support adaptive responses and restoring homeostasis (Certo et al., 2021). Notwithstanding, if the inflammation is perpetuated, alongside angiogenesis and adipose tissue expansion, this condition may result in insulin resistance, fibrosis, adipocyte dysfunction, and cell apoptosis (K. Sun et al., 2011).

Although insulin resistance is commonly accepted as the main feature of MetS, there is evidence to indicate that oxidative stress is intrinsically linked to a state of chronic low-level inflammation and MetS. Excess nutrients in the diet cause oxidative stress, probably due to energy homeostasis disturbance in metabolic tissues, such as the liver and adipose tissue (Catrysse & van Loo, 2017; Fernández-Sánchez et al., 2011; Johnson et al., 2016; H.-M. Liu et al., 2018; Mathis, 2013) (Fig. 4). The TLR signaling pathways appear to be the link between

oxidative stress and inflammation. In addition, it is known that oxidative stress leads to the activation of inflammatory kinases that trigger vascular dysfunction and metabolic alterations (Könner & Brüning, 2012b; Porras et al., 2017b; Silva et al., 2018). In turn, inflammation can also lead to oxidative stress, through superoxide generation, protein kinase C activation, and polyol and hexosamine pathways, among other mechanisms (Johnson et al., 2016). The adipose tissue also responds to oxidative stress, not only inducing adipocyte proliferation and differentiation but also triggering inflammation that can alter insulin signaling, which may influence food intake (Kelly et al., 2016). Furthermore, oxidative stress can mediate the crosstalk between different organs, for example, between the intestine and adipose tissue (Gil-Cardoso et al., 2017). Animals fed with WD showed alterations in permeability genes, ileal inflammation, and loss of multiple glutathione-S transferase isoforms, leading to oxidative stress in the intestine and the adipose tissue (Gil-Cardoso et al., 2017). Therefore, it is of the utmost importance to understand which event will trigger first so that it can be undone, and homeostasis restored.

Metabolic syndrome encapsulates complex and interconnected cellular and biochemical pathways. The most common metabolic parameters used to assess MetS are waist circumference, TAG, and fasting blood glucose levels (K. g. m. m. Alberti et al., 2009; Mameli et al., 2017). In Table 1 there are the diagnostic criteria for MetS according to World Health Organization, International Diabetes Federation, American Heart Association, and European group for the study of insulin resistance. Of the pathological alterations aforementioned, at least three parameters must be altered in MetS: oxidative stress, inflammation, and altered insulin signaling (which is associated with an impaired glucose transport), as well as dysregulated lipolysis, through biomarkers that indicate an altered lipid and carbohydrate metabolism (James et al., 2012; Rodríguez-Correa et al., 2020).

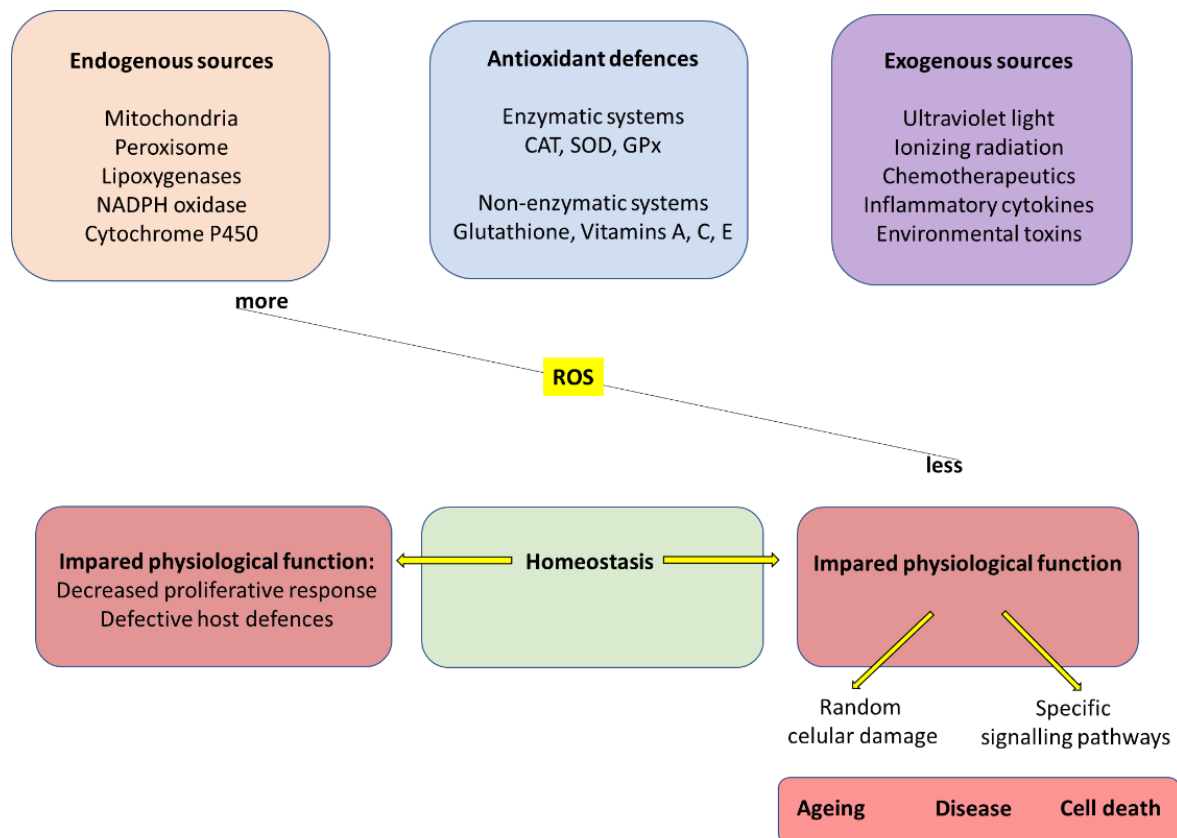


Figure 4 - Sources and cellular responses to reactive oxygen species. Oxidants are generated because of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, several external agents can trigger reactive oxygen species (ROS) production. A sophisticated enzymatic and non-enzymatic antioxidant defense system including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defense. Similarly, increased ROS may also be detrimental and lead to cell death or acceleration in aging and age-related diseases. Traditionally, the impairment caused by increased ROS is thought to result from random damage to proteins, lipids, and DNA. In addition to these effects, a rise in ROS levels may also constitute a stress signal that activates specific redox-sensitive signaling pathways. Once activated, these diverse signaling pathways may have either damaging or potentially protective functions. Adapted from: Toren Finkel, 2000

Table 1 - Diagnostic criteria for metabolic syndrome according to different organizations

World Health Organization				
Required diabetes mellitus, impaired fasting glucose, impaired glucose tolerance, or insulin resistance and at least two of the following:				
Waist-to-hip ratio >0.9 in men, Waist to hip ratio >0.85 in women	TAG >150mg/dL	Blood Pressure >140/90mmHg	Urinary albumin excretion rate >2 mg/g	Albumin to creatinine ratio >30mg/g
International diabetes federation				
Any three of the following:				
Required Waist Circumference by the cutoffs* and at least two of the following:	TAG >150mg/dL	Blood Pressure >130/85mmHg	HDL-C <40mg/dL in men, HDL-C <50 in women	Fasting blood glucose >100mg/dL
American heart association				
Three or more of the following:				
Waist Circumference >40 inches in men, and < 35 inches in women	TAG >150mg/dL	HDL-C <40mg/dL in men, HDL-C <50mg/dL in women	Systolic blood pressure ≥ 130 / 85mmHg	Fasting blood glucose >100mg/dL
European group for the study of insulin resistance				
High-fasting insulin or Insulin resistance and two of the following:				
Waist Circumference ≥94cm in men, and ≥80cm in women	TAG >2mM	HDL-C <1mg/dL	Blood Pressure ≥140/90mmHg	Fasting blood glucose ≥ 6.1mM

* Specific values according to ethnicity, can be consulted at the website: www.idf.org.

1.2 Metabolic syndrome in children and adolescents

Many stages of human development are sensitive to metabolic physiology patterns and are determinants in the immune system template and its response. In MetS there are codependent interactions between inflammation, oxidative stress, and dysbiosis, which have been observed in children and adolescents (Al-Hamad & Raman, 2017). Therefore, unhealthy eating habits during the first years of life contribute to the development of metabolic disorders later in life, which can be explained by metabolic programming that, in turn, may lead to MetS (De Marco et al., 2021; Weihrauch-Blüher et al., 2019).

Childhood obesity increases up to five-fold the risk of obesity later in life and is associated with hypertension, dyslipidemia, and impaired glucose metabolism (Gregory, 2019). Furthermore, dyslipidemia and high blood pressure in children are associated with premature atherosclerosis in adults (Agirbasli et al., 2016). Emerging studies are focusing on the onset of MetS in childhood, in an attempt to prevent the risk of chronic diseases later in life. Evidence suggests that obesity-associated metabolic dysfunction can be framed as a childhood disease in which growth, development, environment, and genetic predisposition are interlinked (Negrea et al., 2021). This framework provides a new perspective on the potential mechanisms by which inflammation and metabolism interact throughout life and could change the treatment and prevention of metabolic diseases.

For anthropometric parameters assessment, BMI has been used as a measurement of body fat for children the age of 2 years or more, in opposition to the simple evaluation of the excess of adipose tissue. BMI has its limitations. It does not, for example, differentiate fat from muscle or differences across the bone structure. Hence, it is more accurate to use an anthropometric index associated with central obesity. Waist circumference measurement is a representative index of central obesity, and evidence suggests an association between waist circumference and obesity-related morbidity (Trandafir et al., 2020). Central obesity is a risk factor for chronic metabolic disease development, such as T1DM, that may persist from childhood into adulthood (Agirbasli et al., 2016; Cook, 2004; Goodman et al., 2004).

The definition of MetS in children and adolescents is based on the definition of MetS for adults, but there is no consensus on the absolute criteria to assess risks or outcomes

(Higgins & Adeli, 2017). Therefore, it is necessary for a simple and unified clinical definition to early identify MetS in young people globally (Fig. 5).

MetS in Children and Adolescents

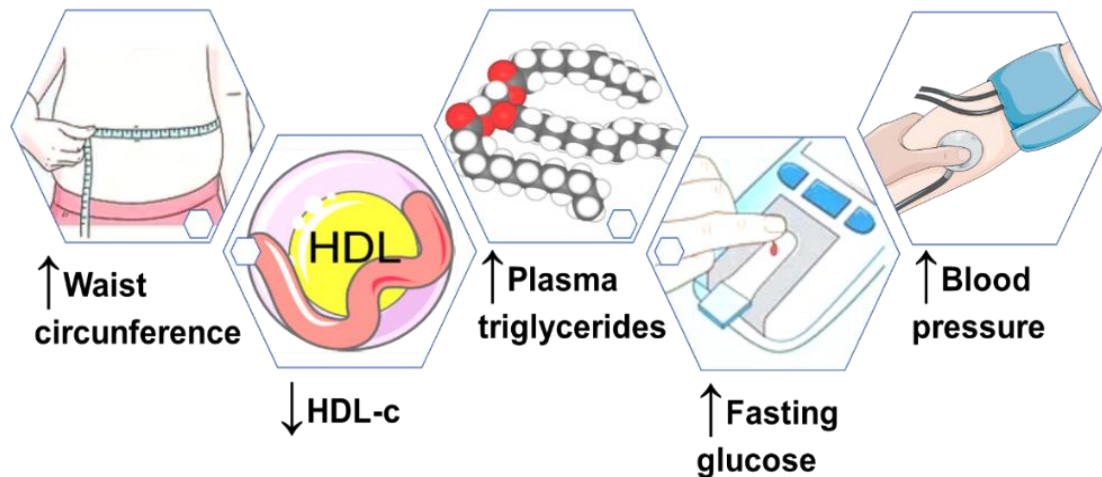


Figure 5 - Criteria for clinical diagnosis of metabolic syndrome in childhood and adolescence. The definition of metabolic syndrome in this age group is central obesity plus the presence of two or more two components. HDL-C: high-density lipoprotein cholesterol. Adapted from H. H. Wang et al., 2020

The International Diabetes Federation's definition of the at-risk group and the MetS in children and adolescents are shown in Table 2. It has been established, based on modifications of previous adult standards, and it requires the presence of central obesity plus any two of the other four factors: elevated TAG, low HDL-C, high blood pressure, and high plasma glucose (Zimmet et al., 2007).

Table 2 - The International Diabetes Federation's definition of the at-risk group and the metabolic syndrome in children and adolescents

Age group: 6 – <10* years				
Obesity – Waist Circumference Percentile: ≥90th				
Age group: 10–<16 years				
Obesity – Waist Circumference ≥90th percentile or adult cut-off if lower	TAG ≥1.7mmol/L (≥150 mg/dL)	HDL-C <1.03 mmol/L (<40 mg/dL)	Blood Pressure (BP) Systolic BP ≥130 or Diastolic BP ≥85 mmHg	Plasma glucose Fasting Plasma Glucose ≥5.6 mmol/L (100 mg/dL) [‡] or known TIID
Age group: Adult criteria >16				
Obesity – Waist Circumference ≥94 cm for Euroid males and ≥80 cm for Euroid females, with ethnic-specific values for other groups [†]	TAG ≥1.7mmol/L (≥150 mg/dL) or specific treatment for high TAG	HDL-C <1.03 mmol/L (<40 mg/dL) in males and <1.29 mmol/L (<50 mg/dL) in females, or specific treatment for low HDL-C	Blood Pressure (BP) Systolic BP ≥130 or diastolic BP ≥85 mmHg or treatment of previously diagnosed hypertension	Plasma glucose Fasting plasma glucose ≥5.6 mmol/L (100 mg/dL) [‡] or known TIID

WC: waist circumference, BP: blood pressure.

[†]For those of South and South-East Asian, Japanese, and ethnic South and Central American origin, the cut-offs should be ≥90 cm for men, and ≥80 cm for women. The International Diabetes Federation Consensus Group recognizes that there are ethnic, gender, and age differences, nonetheless, future research is needed on outcomes to establish the risk.

[‡]If fasting plasma glucose is 5.6–6.9 mmol/L (100–125 mg/dL) and it is not known to have diabetes, an oral glucose tolerance test should be performed.

It is urgent and imperative to prevent the onset of MetS in children. Most of the approaches nowadays, take a path of lifestyle change with alterations in eating habits and energy expenditure. The success of lifestyle changes depends on compliance by those targeted. Because they are imposed and not understood and accepted, it explains why in many situations they fail in the long run. Selected cases, pharmacological interventions, and bariatric surgery have already been considered.

The rigid cut-off points of MetS definitions do not have into consideration physiological fluctuations, known for a transitional reduction of insulin sensitivity (Goran & Gower, 2001). At the burst of puberty, it was also documented some transient changes in the cardiovascular risk factors (Reinehr, 2016), that undermine the reliability of a universal MetS definition for both stages, before and after puberty. The diagnosis of pediatric MetS currently does not help to predict future consequences in these individuals, due to hormonal changes related to puberty. Cardiometabolic risk assessment in pediatrics should be based on risk factors such as hypertension, dyslipidemia, insulin resistance, and nutritional status (Samson & Garber, 2014).

1.3 Diet and metabolic homeostasis

In recent years, weight problems and obesity are increasing at a rapid rate and the prevalence of MetS has therefore increased (Wang et al., 2020) (Fig. 6). On the other hand, it is well established that unhealthy dietary patterns are the main drivers of MetS development. As complex carbohydrates are digested more slowly, they are recommended (Astrup & Hjorth, 2018). In turn, simple carbohydrates, especially refined carbohydrates such as sucrose or other added sugars, should be limited (Bailes et al., 2003; Foster et al., 2003). Excessive consumption of sugary beverages constitutes one of the most common sources of added sugars, in addition to fruits, sweets, cakes, dairy desserts, and pies (Dietz, 2006; Guthrie & Morton, 2000). Although the chemical structure is identical in added and simple sugars, added sugars are nutritionally poor, containing almost no vitamins, minerals, or fiber. Added sugars have a more expressive glycaemic effect, raising glucose levels quickly, and changing the development of insulin resistance. Based on their glycaemic index, sugars can be classified as

good sugars or bad sugars (Dowla et al., 2018; Hall & Chung, 2018). As hypercaloric diets have a high amount of dietary energy available they are commonly implicated in the MetS. However, it is noteworthy the effect different levels of sucrose also have on the development of the MetS.

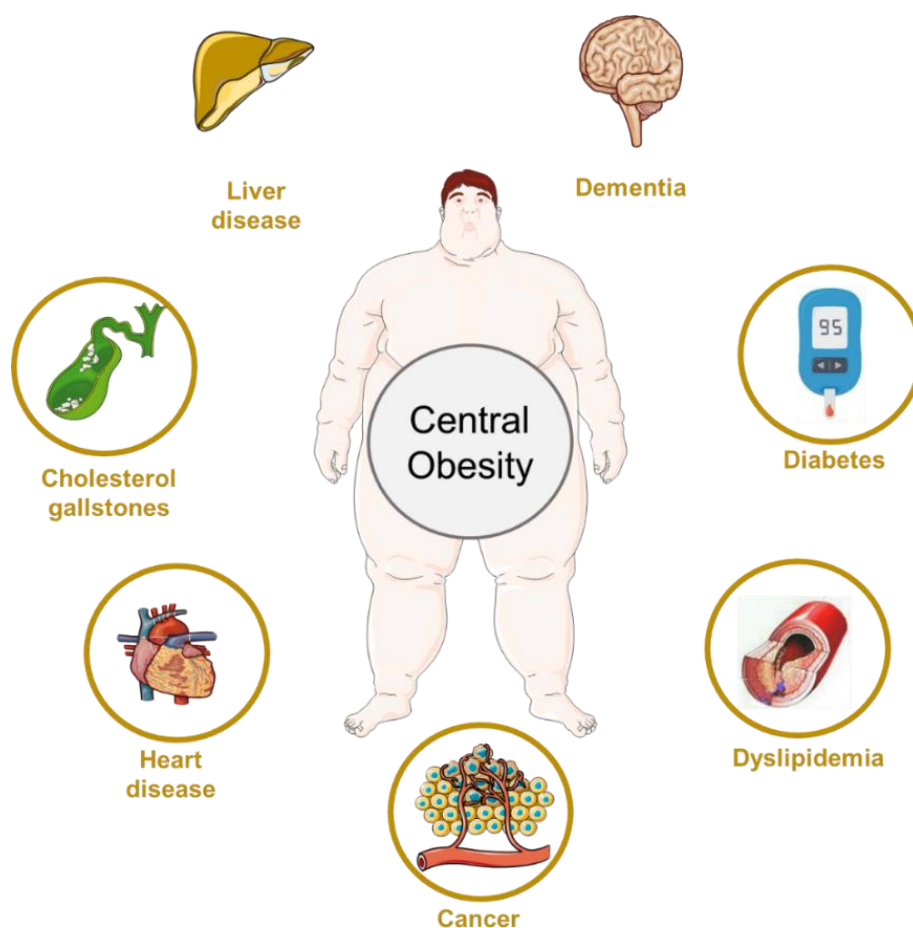


Figure 6 - Obesity and insulin resistance play a key role in the pathogenesis of metabolic syndrome in childhood and adolescence. Clinical and epidemiological studies demonstrated that central obesity is often associated with several metabolic abnormalities like impaired glucose tolerance, insulin resistance, hypertension, hypercholesterolemia, and hypertriglyceridemia. Metabolic syndrome is the cluster of these metabolic abnormalities and is associated with an increasing prevalence of metabolic diseases such as T1D and CVD. Adapted from H. H. Wang et al., 2020 _This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; <https://smart.servier.com>.

Unhealthy dietary patterns, such as WD, are characterized by the consumption of ultra-processed foods, which are low in fiber, high in calories, cholesterol, saturated fatty acids, refined carbohydrates, and salt content (Conlon & Bird, 2014; Danaei et al., 2013; Wang et al., 2008). Currently, there is excessive consumption of fast food, which, alongside a low

level of physical activity, promotes metabolic abnormalities such as obesity, dyslipidemia, hypertension, T1D, and CVD in children and adolescents (Cordain et al., 2005). The adoption of the Mediterranean diet, with or without caloric restriction, contributes to the normalization of the lipid profile, with an increase in the intake of unsaturated fat, mainly from olive oil, consumption of legumes, whole grains cereals, fruits, vegetables, nuts, fish, and low-fat dairy products (Pacifico et al., 2011; Pérez-Martínez et al., 2017). The Mediterranean diet reduced the prevalence of MetS by 50% (Kastorini et al., 2011), besides beneficial effects on its most common conditions, such as abdominal obesity, dyslipidemia, high fasting glucose, and high blood pressure (Pérez-Martínez et al., 2011).

A proper diet and good nutrition status are considered crucial factors in life course development (Moore et al., 2018). The brain's metabolic activity relies on oxidative phosphorylation and, normally, responds to bioenergetic challenges as a way of maintaining homeostasis (Camandola & Mattson, 2017). Therefore, an accumulation of oxidative stress damage throughout life can be one of the key mechanisms for brain health or dysfunction (Chakrabarti et al., 2011). Several dietary components, acting as antioxidant, anti-inflammatory, and/or insulin action potentiating factors, could positively participate in a nutritional strategy for the healthiness of brain functions. Evidence has shown that n-3 polyunsaturated fatty acids (PUFAs) have beneficial effects on the regulation of cellular redox homeostasis, suggesting their potential ability to protect cognitive function (Poulose et al., 2017; Sun et al., 2018). One of the PUFAs, docosahexaenoic acid (DHA), undergoes enzymatic conversion to form oxylipins, with anti-inflammatory and vasodilatory properties (Sun et al., 2018). Other nutrients with antioxidant properties have also been shown to protect brain cells from oxidative stress such as flavonoids (Vauzour et al., 2017). So it is not surprising that brain dysfunction most often co-occurs with metabolic disorders and/or unhealthy dietary patterns (Moore et al., 2018). For example, adherence to a Mediterranean diet seemed to preserve brain health (Moore et al., 2018; Vauzour et al., 2017) while obesogenic diets were associated with negative health implications including cognitive dysfunctions (Esteban-Cornejo et al., 2018; Taylor et al., 2017). The correlation between the Mediterranean diet and brain health is associated with a higher intake of several ingredients or nutrients, such as dietary fiber, low-glycemic carbohydrates, vitamins, PUFAs, and polyphenols (Poulose et al., 2017).

Finally, diet is a key modifiable factor influencing the composition of the gut microbiota (GM). Therefore, the link between metabolism, oxidative stress, and inflammation might lie at the functional interface of cells of primarily immune or metabolic nature, such as adipocytes, gut, liver, and macrophages, but also on the crosstalk between GM and intestinal epithelial cells.

1.4 Metabolic syndrome and the gut-brain axis

The study of GM-host interactions is a rich area of investigation, as it is involved in both physiology and pathology, having a relevant role in neuroendocrine functions, energy metabolism, and in the regulation of the immune system, which may reveal new strategies for the prevention of MetS and its complications (Fig.7) (Shen et al., 2013). Although the physiological and environmental predispositions underlying MetS are not yet fully understood, it is now known that GM and intestinal permeability can be central pieces at the beginning of MetS, interfering with several functions of the host, including energy and immune regulation and resistance to infection (Bäckhed et al., 2004; Cani et al., 2008).

Dietary habits and obesity-induced low-grade inflammation appear to cause an impaired intestinal barrier function and disruption of the host energy homeostasis (Fig.7) (Birchenough et al., 2015). For the normal coexistence of commensal microbial communities and mucosal immune cells, the integrity of the intestinal epithelium is important to the maintenance of the host physiology, as it prevents the paracellular translocation of bacterial components (Leser & Mølbak, 2009; Neish, 2009; Sansonetti & Medzhitov, 2009). The mucus layer that covers the intestinal epithelium is produced by goblet cells, acting as a protective layer of the intestine mucosa, and can respond to stimuli in a region-dependent manner (Birchenough et al., 2015). Abnormal changes in the mucus layer can be a consequence of pathological conditions that occur in the intestine barrier, for instance, a high-fat diet can influence goblet cells to produce less mucus, an effect that occurs even before metabolic alterations or obesity (Specian & Oliver, 1991).

Diet composition plays an important role in the control of GM communities, particularly in early life. Because dietary fiber is the main energy source for several bacteria,

they affect mucin glycan production and mucus barrier integrity and, thus, affects microbiota composition (De Marco et al., 2021). In turn, mucin glycan is used as a nutrient by bacteria with the concomitant expansion of some bacterial genera, causing erosion of the mucus barrier and altering intestinal permeability, but also decreasing beneficial microbiota-derived metabolites, such as short-chain fatty acids (Malesza et al., 2021). Also, the consumption of dietary saturated fats and added sugar is linked to the alteration of the colonic microbiota composition and the onset of inflammation-associated pathways, and colonic dysmotility (Nascimento et al., 2021). The intestinal barrier is affected on one hand, by a high-fat diet, differently in the ileum and the colon (Nascimento et al., 2021; Oliveira et al., 2019). On the other hand, high-sugar beverages induce alteration of GM and impairment of gut epithelium integrity, leading to a systemic inflammatory response (Do et al., 2018) and alterations in glucose homeostasis (Ralston et al., 2017; Vandanmagsar et al., 2011).

As diet plays an essential role in the development and stability of GM; dysbiosis related to the consumption of unhealthy diets arises as a new concern to face the challenges of global obesity (De Marco et al., 2021). Dysbiosis refers to the decrease or loss of the host beneficial bacterial communities in the GM and, the concurrent expansion of pathobionts, leading to various pathological manifestations in the gastrointestinal tract and systemically (König et al., 2016; Rohr et al., 2018). An increase in intestinal permeability, results in circulating levels of LPS from intestinal bacteria, directly correlated with the onset of T1D (Shi et al., 2006) and with the potential for activating pattern recognition receptors and TLR4 in metabolic organs such as liver and adipose tissue (Amar et al., 2011). Studies with children and adolescents have shown that the translocation of bacterial endotoxin from the gastrointestinal tract causes a pro-inflammatory response (Pussinen et al., 2011; Vael & Desager, 2009). In addition, this endotoxemia has been associated with an increase in blood pressure, arterial dysfunction, also, with alterations of the lipid profile (Brown et al., 2012; Chang et al., 2015; Kheirandish-Gozal et al., 2014).

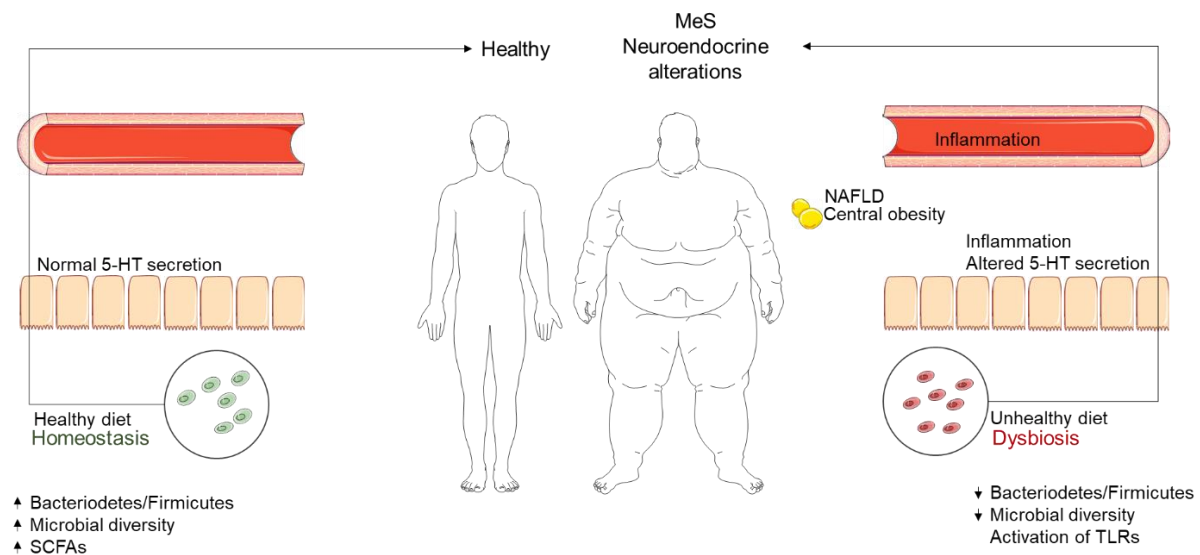


Figure 7 - Interplay between diet, metabolic syndrome (MetS), and the gut-brain axis. A healthy dietary pattern contributes to epithelial gut integrity, immune homeostasis, and a “normal” GM, that helps regulate the gut-brain axis. Unhealthy diets reduce microbial diversity, promote inflammation, NAFLD, and central obesity, and promote the “leaky gut”, favoring the translocation of LPS from Gram-negative bacteria, increasing inflammation and alterations in the CNS. Dysbiosis induced by diet is linked to central obesity, due to its adverse consequences on local and systemic inflammation, leading to neuroendocrine dysregulation. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; <https://smart.servier.com>.

In children, endotoxin levels induced by diet and central obesity can define the interventions needed to reduce the risk of MetS onset and its progression into adulthood (Kellow et al., 2014; Lira et al., 2010). At birth, the GM of a child is sterile, but it is in an adult-like state by 12 to 36 months (Laue et al., 2022). In the early years of life, strong shreds of evidence indicated that GM can greatly influence many bio-behavioral aspects, such as brain function and stress responses through the gut-brain axis, in which bacterial communities play a key role in neuro-immune system's complex regulatory functions during early life (Cong et al., 2015). Furthermore, infancy and early childhood present higher vulnerability to the effects of diet, but they are also a strong driver of the GM composition (Turnbaugh et al., 2009).

GM emerges as an important player in the gut-brain axis, which can provide insights into the interplay between energy metabolism, brain function, and behavior (Dinan & Cryan, 2017; Sampson & Mazmanian, 2015). GM can influence the enteric nervous system activity, for example, the afferent nerves, by producing or inducing molecules that can act as local neurotransmitters, such as gamma-aminobutyric acid (GABA), serotonin, and acetylcholine (Huang & Wu, 2021). Moreover, it produces a wealth of neuroactive substances such as

catecholamines, histamine, and other compounds, like endocannabinoids (eCBs)(Minichino et al., 2021). For example, the endocannabinoid system family (i.e. cannabinoid receptors, eCBs, and respective metabolic enzymes), commonly associated with the central nervous system, are present in gut epithelium and enteroendocrine cells. The eCBs regulate gut motility, permeability, and inflammatory responses. In turn, GM has been shown to influence the activity of the endocannabinoid system (Srivastava et al., 2022). Furthermore, the eCBs signaling mechanisms have been proposed to participate in the control of food intake and energy balance both indirectly through circulating gut-derived factors and directly through the vagus nerve. Additionally, some studies suggest that eCBs signaling within the gut plays a key role in driving the intake of dietary fat due to its distinguishable taste properties (DiPatrizio et al., 2013).

The neuroactive substances produced by GM ensure the proper maintenance of gastrointestinal homeostasis, but also seem to have effects on higher cognitive functions. For example, some studies suggest a causal link between GM and features of mental disorders, including anhedonic/amotivational behaviors (Zheng et al., 2019). In addition, it was shown that GM plays a role in the promotion of gut and blood serotonin levels, raising its synthesis by enterochromaffin cells (Choi et al., 2018). Enteric serotonin is an important immunomodulator, that regulates leptin production and influences liver regeneration (Oh et al., 2015). Immune cells express receptors for serotonin, suggesting bi-directional signaling in the immune-endocrine axis, and for so, it might be considered that the immune response and metabolic regulation influence each other (Jenkins et al., 2016; Reigstad et al., 2015). Nevertheless, more research is needed to unravel the influence of dietary factors on the crosstalk between GM and host metabolism, particularly in early life.

2 Aims

This research thesis aimed to investigate the long-term effects of unhealthy eating habits, in young male rats, exposed to a high-sugar diet (HSD) and WD, specifically on immunometabolic response, oxidative stress, and endocrine system. The following specific aims were established:

- 2.1 Compare metabolic profile differences from both HSD and WD diets. Determine the plasmatic concentration of glucose, creatinine, urea, total proteins, and enzymes such as alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST), as well as the lipid profile and leptin levels.
- 2.2 Morphologically characterize HSD and WD diet effects on the gastrointestinal tract, liver, and adipose tissue. To evaluate the effects of different diets on the gut, liver, and adipose tissue morphology, histomorphometric analysis was performed on tissue sections.
- 2.3 Evaluate HSD and WD diet effects on the digestive tract, liver, and adipose tissue inflammation and oxidative stress. To study the effects of both diets on the inflammatory profile, the hepatic expression of TLR4, COX-2 and E-Selectin were determined as well as lipid peroxidation. In the small intestine and colon, expression of mRNA levels of iNOS TLR2, TLR4, NFκB, and NLR3 were assessed.

3 Publications

3.1

Early unhealthy eating habits underlie morpho-functional changes in the liver and adipose tissue in male rats

[Sofia Nogueira](#), [Fernanda Garcez](#), [Susana Sá](#), [Luís C Moutinho](#), [Armando Cardoso](#), [Raquel Soares](#), [Bruno M Fonseca](#), [Sandra Leal](#)

Histochem Cell Biol. 2022 Jun;157(6):657-669 (doi: 10.1007/s00418-022-02092-2)

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3.2

Unhealthy diets induced distinct and regional effects on intestinal inflammatory signaling pathways and long-lasting metabolic dysfunction in rats

[Sofia Nogueira](#), [Joana Barbosa](#), [Juliana Faria](#), [Susana Sá](#), [Armando Cardoso](#), [Raquel Soares](#), [Bruno M Fonseca](#), [Sandra Leal](#)

International Journal of Molecular Sciences 2022 Sep; 23.18: 10984 (doi.org/10.3390/ijms231810984)

3.1

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Early unhealthy eating habits underlie morpho-functional changes in the liver and adipose tissue in male rats

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Abstract

Early-life consumption of high-fat and sugar-rich foods is recognized as a major contributor for the onset of metabolic dysfunction and its related disorders, including diabetes and nonalcoholic fatty liver disease. The lifelong impact of early unhealthy eating habits that start at younger ages remains unclear. Therefore, to better understand the effects of diet, it is essential to evaluate the structural and functional changes induced in metabolic organs and potential mechanisms underlying those changes. To investigate the long-term effects of eating habits, young male rats were exposed to high-sugar and high-energy diets. After 14 weeks, body composition was assessed, and histopathological changes were analyzed in the liver and adipose tissue. Serum biochemical parameters were also determined. Expression of inflammatory markers in the liver was evaluated by immunohistochemistry. Our results revealed that serum levels of glucose, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), and lipid profile were increased in rats red high-sugar and high-energy diets. Histopathological alterations were observed, including abnormal hepatocyte organization and lipid droplet accumulation in the liver, and abnormal structure of adipocytes. In both unhealthy diet groups, hepatic expression of Toll-like receptor 4 (TLR4), cyclooxygenase 2 (COX-2), and E-selectin were increased, as well as a biomarker of oxidative stress. Together, our data demonstrated that unhealthy diets induced functional and structural changes in the metabolic organs, suggesting that proinflammatory and oxidative stress mechanisms trigger the hepatic alterations and metabolic dysfunction.

Keywords High-energy diet · High-sugar diet · Young rat model · Metabolic dysfunction · Immunohistochemistry · Histomorphometric study

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Introduction

Metabolic syndrome is becoming increasingly prevalent in Western society as a result of sedentary lifestyles and dietary patterns (Lee and Yoon 2018; Weihe and Weihrauch-Blüher 2019). It refers to a combination of metabolic disturbances, including insulin resistance, dyslipidemia, glucose intolerance, obesity, and hypertension (Weihe and Weihrauch-Blüher 2019). In childhood, an excessive consumption of sweetened beverages and calorie-dense foods is associated with increased risk of persistent obesity and adult obesity-related complications (Lee and Yoon 2018).

The pathophysiology underlying the metabolic alterations induced by unhealthy diets is complex and may involve multiple interrelated mechanisms. Overfeeding induces oxidative stress (OS) as it is linked to altered oxygen metabolism and chronic low-grade inflammation. OS can be caused by the disruption of energy homeostasis in several metabolic tissues such as liver and adipose tissue (Fernández-Sánchez et al. 2011; Mathis 2013; Johnson et al. 2016; Catrysse and van Loo 2017; Liu et al. 2018). The mechanisms by which inflammation is triggered remain uncertain, although it is well documented that excess nutrient availability causes cellular degeneration and activation of inflammatory pathways through Toll-like receptors (TLRs) (Mathis 2013; Christ et al. 2019). In turn, the inflammatory response is orchestrated by proinflammatory cytokines through a TLR signal-dependent manner (Liu et al. 2017, 2018). Free fatty acids can bind to TLR4 and TLR2 resulting in nuclear factor (NF)- κ B and c-Jun N-terminal kinase 1 (JNK1) activation, triggering inflammatory pathways (Könner and Brüning 2011). NF- κ B is the transcription factor involved in these inflammatory reactions that also transcriptionally controls cyclooxygenase 2 (COX-2) expression (Sol and Fresno 2005). Eicosanoids generated from COX-2 activation are important players in the manifestation of inflammatory pathology by increasing the synthesis of adipocytes and hepatocytes and the secretion of chemokines. Consequently, infiltration of proinflammatory macrophages occurs (Rogerio and Calder 2018) (Hardwick et al., 2013).

Nonalcoholic fatty liver disease (NAFLD) has emerged as one of the most common chronic liver diseases in Western society (Cotter and Rinella 2020; Peng et al. 2021). NAFLD is a hepatic manifestation of metabolic syndrome, being related to an excessive caloric intake and genetic predisposition, ranging from a simple increase of intrahepatic lipid content to nonalcoholic steatohepatitis (NASH) with inflammation, fibrosis, and cirrhosis (Cotter and Rinella 2020; Peng et al. 2021).

Experimental studies of diet-induced obesity and metabolic syndrome commonly use rodent models feeding with

commercial high-fat diets (HFD) (Kennedy et al. 2010), which cannot be extrapolated to humans' eating habits. Nonetheless, in the present study rats received the high-energy diet as it reflects more accurately the unhealthy human eating behaviors, namely the high consumption of sugary drinks and palatable and processed foods by children and adolescents in Western society. The intake of beverages with a high content of simple sugars does not always accompany a high fat intake and for so, it is a current model for metabolic syndrome studies, per se. Preclinical research can be helpful to understand the detrimental effects of consumption of high-sugar and high-energy diets at younger ages, namely their association with metabolic syndrome and target organ damage. With this study, we intended to study the alterations of the structure and function of metabolic organs and possible mechanisms which may underpin these effects. Since metabolic disease at younger ages can have a profound impact on public health, it is crucial to mitigate the risk of chronic diseases in adulthood.

Material and methods

Animals and experimental procedures

Twenty-seven male Wistar rats obtained from the colony of the Institute for Molecular and Cell Biology /i3S (Porto, Portugal) were bred and raised at the main animal facility of the Faculty of Medicine, University of Porto. They were kept in a controlled environment, housed in a clean facility, under a 12:12 h light/dark cycle. Animals were housed two per cage according to their weight, since there is evidence that rearing in social isolation induces endocrine, physiological, and behavioral changes (Weiss et al. 2004). Throughout the study, animals had ad libitum access to liquid and solid food, which was replaced daily. At postnatal day 21–23, rats were randomly assigned to three dietary treatments ($n=9$ per diet group) for 14 weeks. Control diet group (C) was fed with standard rat chow (4RF21/C Mucedola, Italy) and had free access to water. The high-sugar diet group (HS) was fed standard chow diet and had free access to 30% sucrose solution (Sigma–Aldrich Company Ltd., Madrid, Spain; 1.2 kcal/ml). The high-energy diet group (HED) was fed standard chow diet mixed with a selection of palatable human foods (chocolate cake, biscuits), dog roll, and fat, and had free access to 15% sucrose solution (Sigma–Aldrich Company Ltd., Madrid, Spain; 0.6 kcal/ml). The compositions of the solid diet were as follows: the chow diet contained 3.9 kcal/g with 20% protein, 12% fat, and 68% carbohydrates. The HED contained an average of 4.5 kcal/g, approximately 12% protein, 45% fat (38% from saturated fat), and 43% carbohydrates (40% from sucrose).

The amount of solid and fluid intake was calculated daily, and the rats were weighed weekly. The average daily energy intake (Table 1) was estimated from the diet macronutrient composition based on the Atwater factors, assigning 4, 4, and 9 kcal/g for the available carbohydrate, protein, and fat, respectively.

Experiments were performed according to the national and international guidelines and with approval of local authorities, Animal Care and Use Committee (ORBEA) of the Faculty of Medicine of the University of Porto (Portugal). All the procedures were performed by licensed users of Federation of Laboratory Animal Science Associations (FELASA) and in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU).

Tissue and blood collection

Animals were anesthetized with sevoflurane (SevoFlo, Abbott Laboratories Ltd, Maidenhead, UK). Blood samples were drawn by cardiac puncture, using a heparinized needle. The serum was separated by centrifugation at 4 °C (Heraeus Biofuge Pico, Hanau, Germany), 2000 rpm for 15 min and stored at –80 °C. Liver and adipose tissues (perigonadal and abdominal adipose tissues) were isolated, collected, and weighed. The total adipose tissue weight was determined by sum of perigonadal and abdominal adipose tissue weights. The adiposity index was the total adipose tissue weight per 100 g of body weight (gbw). The percentage of perigonadal and abdominal adipose tissue was also calculated. A sample of liver and adipose tissue were post-fixed in 10% formalin dissolved in PBS, pH 7.4 and processed for routine histology analysis. The remaining of liver and adipose tissues were rapidly frozen and stored at –80 °C for further analysis.

Serum biochemical and hormonal measurements

Biochemical parameters were determined directly in serum, using the Prestige 24i automated analyzer (Cormay, Tokyo

Boeki) as previously described (Barbosa et al. 2017). The concentrations of glucose, creatinine, urea, total proteins, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c), alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) were quantified. Enzyme activities were determined as U/L and other biochemical parameters were retrieved as mg/dL; all reagents were purchased from Cormay (Marynin, Poland). For each parameter, calibrations were performed with two appropriate calibrators to plot a standard curve with five points. Leptin concentrations (as ng/mL) were quantified by ELISA (Abcam, Cambridge, UK), using a commercial kit and performed according to the manufacturer's protocols.

Liver and adipose tissue histomorphology

Paraffin-embedded liver tissue samples were sectioned at 4 µm on a microtome. One set of sections was stained with hematoxylin and eosin (H&E) (Diapath, Italy) for evaluation of general histology; other sets were stained with Masson trichrome (Sigma–Aldrich, Taufkirchen, Germany) and Sirius Red (Abcam, Cambridge, UK) for qualitative and quantitative evaluation of hepatic fibrosis, and Sudan Black B (Merck, Darmstadt, Germany) staining was used to assess lipid droplet distribution. Sections were observed using a Nikon Eclipse E600 microscope and photomicrographs were acquired with a Nikon Ds-Ri2 digital camera. Hepatic fibrosis was quantified in Sirius Red-stained sections using ImageJ software (<http://rsb.info.nih.gov/ij/>; freely available from the National Institutes of Health, Bethesda, MD, USA). Morphometric analyses were performed in photomicrographs of 5–8 optic fields randomly selected per rat, and the result was expressed as area in square micrometers. Paraffin-embedded adipose tissue samples were sectioned at 4 µm and stained with H&E to evaluate general histology. To quantify the adipocyte area, ImageJ software was used. The

Table 1 Daily energy intake and energy contributions of macronutrients

Average daily energy intake (kcal/day/rat)	Control diet	High-sugar diet	High-energy diet
Total energy intake	69 (2)	82 (3)	152 (5)
From solid food	69 (2)	30 (2)	123 (7)
Protein	13.7 (0.4)	6.1 (0.4)	14.8 (0.8)
Carbohydrates	46.7 (1.5)	20.7 (1.5)	53.1 (2.9)
From sucrose	–	–	21.2 (1.2)
Lipids	8.2 (0.3)	3.7 (0.3)	55.5 (3.0)
From saturated lipids	0.11 (0)	0.05 (0)	21.1 (1.2)
From sucrose solution	–	51 (2)	29 (3)

Data is expressed as mean (SD); *n* = 9 per group

mean adipocyte area was determined in digital images of 5–8 optic fields randomly selected and a minimum of 30 adipocytes per animal were measured.

Immunohistochemistry

Liver tissue sections were also processed for immunohistochemistry, as described previously (Leal et al. 2008). Briefly, after dewaxing, hydration, and antigen retrieval in citrate buffer solution, paraffin tissue sections were treated with 3% H₂O₂ for 10 min to inactivate endogenous peroxidase and blocked for nonspecific protein binding with normal goat serum. Sections were incubated in goat polyclonal anti-COX-2 (1:500; #SC1747), TLR4 (1:100; #SC293072), or E-selectin (1:100; #SC137054), overnight. All primary antibodies were purchased from Santa Cruz Technology (CA, USA). After washing with phosphate buffer, the sections were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (BA-1000-1.5, Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:50, for 30 min at room temperature. Afterwards, sections were treated with the avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:800, for 1 h at room temperature. The immunoreaction was visualized after incubation for 3 min, in 0.05% diaminobenzidine (Sigma–Aldrich Ltd., Madrid, Spain) to which H₂O₂ was added to a final concentration of 0.01%. Counterstaining was performed in Mayer's hematoxylin (Sigma–Aldrich). To minimize the variability in staining, sections from all groups analyzed were processed in parallel at the same time, for the same antibody, and appropriate positive (spleen) and negative (in the absence of the primary antibody) controls were used in each test. Photomicrographs of 5–8 randomly chosen fields per animal were taken using a digital camera (DS-Ri2, Nikon) and the Nikon Eclipse E600 microscope.

The expression of TLR4 and COX-2 was semiquantitatively analyzed on the basis of positive staining, under low power magnification, using the following score: negative cases, either no immunoreactivity at all or only occasional expression (less than half the fields had evidence of expression and, when present, expression was sparse) or 1–10 cells/field; positive cases, more than 10 cells/field identified. The percentage of immunoreactivity was calculated, considering the number of positive cases and the number of total fields analyzed. For endothelial expression of E-selectin, it was considered that a positive case has more than five positive stained vessels counted per field (Lazaris et al. 1999), and percentage of positive cases was calculated, considering the number of positive cases and the number of total fields analyzed.

Lipid peroxidation evaluation in the liver

Lipid peroxidation was determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a colorimetric product, proportional to the MDA present, using a commercial kit (Merck, Darmstadt, Germany) and performed according to the manufacturer's protocols. Briefly, liver samples (10 mg) were homogenized on ice in 300 µL of the MDA Lysis Buffer containing 3 µL of BHT. The samples were centrifuged at 13,000×g for 10 min to remove insoluble material. To form the MDA–TBA adduct, 600 µL of the TBA solution was added into each vial containing standard and sample and incubated at 95 °C for 60 min. The samples were cooled to room temperature in an ice bath for 10 min and 200 µL from each reaction mixture were pipetted into a 96-well plate for analysis. The absorbance at 532 nm (A₅₃₂) was measured. The values obtained from the appropriate MDA standards were used to plot a standard curve. The amount of MDA present in the samples was determined from the standard curve. Concentration of MDA is expressed as nanomoles per milliliter.

Statistical analysis

Data is expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to compare the differences among groups, following by Tukey's multiple-comparison tests. Statistical analysis was performed with Graph Pad Prism 4 (Graph Pad Software Inc., San Diego, CA, USA). A *p* value less than 0.05 was considered as statistically significant.

Results

Body weight, liver and adipose tissue weights

There was no significant difference in total body weight between groups at the beginning (week 0) and at the end (week 14) of the dietary intervention (Fig. 1a). There was no difference between the total weight of livers from animals submitted to the different diets, but the total weight of the adipose tissue showed an increase in HED when compared to control diet and HS groups (Fig. 1b).

The effect of dietary intervention on weight of perigonadal, abdominal, and total adipose tissue per 100 g of body weight is shown in Fig. 1c, revealing an increase in HS and HED in all analyzed parameters (*p* < 0.05).

Biochemical and hormonal measurements

Adipocytokines play an important role in regulating lipids and glucose metabolism, and leptin dysregulation has been associated with increased obesity and metabolic disturbances

Fig. 1 Body composition and serum leptin levels in different diet-fed rat groups. **a** Body weight of rats at the beginning (week 0) and the end of dietary intervention (week 14). **b** Average of liver and total adipose tissue weights. **c** Relative perigonadal, abdominal, and total adipose tissue weights expressed per 100 g of body weight (gbw). **d** Serum leptin levels in the different groups. C control diet group, HED high-energy diet group, HS high-sugar diet group. Data is expressed as mean $\pm$ SD. * p < 0.05 vs C group. § p < 0.05 HS vs HED

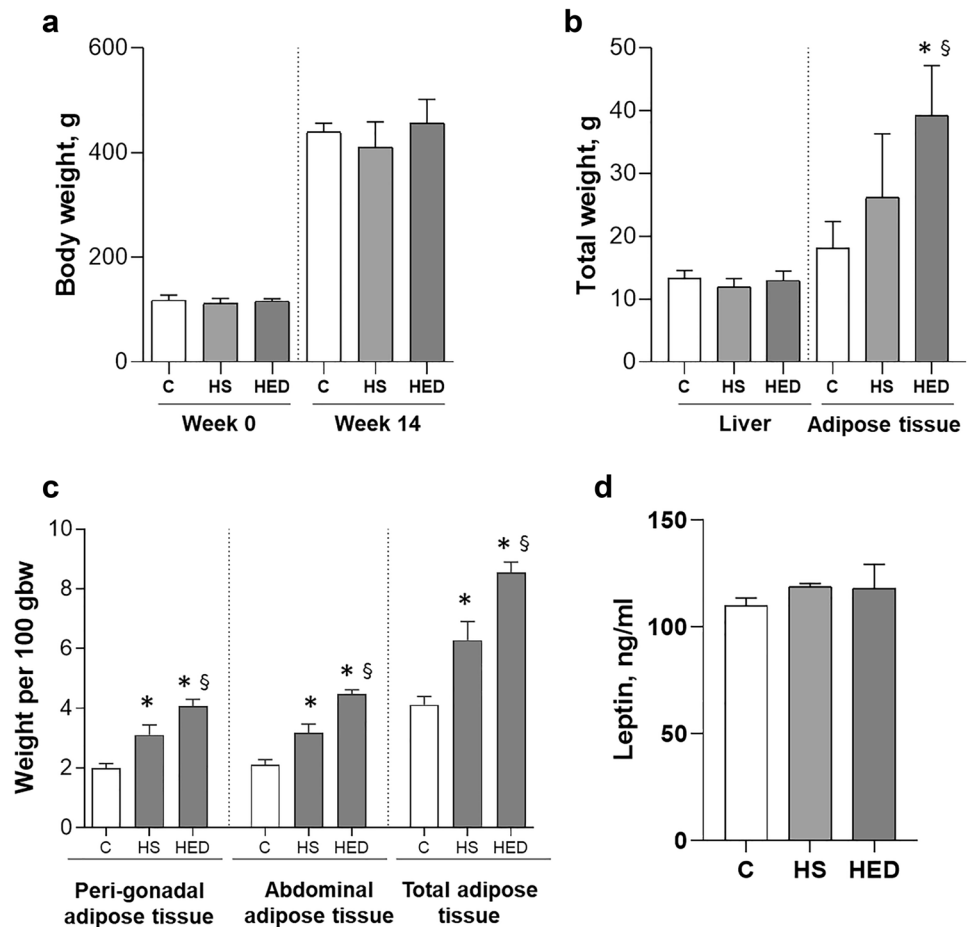


Table 2 Serum biochemical parameters for the control, high-sugar, and high-energy diets

	Control diet	High-sugar diet	High-energy diet
Glucose (mg/dL)	102.3 (8)	186.4 (47)***	204.8 (58)***
Urea (mg/dL)	34.2 (19)	44.3 (14)	52.1 (18)
Total protein (g/dL)	11.0 (0.4)	11.9 (0.1)	11.6 (0.6)
Creatinine (mg/dl)	0.40 (0.2)	0.84 (0.3)*	0.85 (0.4)*
Triglycerides (mg/dL)	120.3 (37)	175.10 (52)	241.3 (80)***
Total cholesterol (mg/dL)	63.8 (7)	86.1 (17)	96.5 (27)**
HDL-c (mg/dL)	20.4 (6.5)	16.2 (3.8)	15.4 (5.2)
LDL-c (mg/dL)	19.4 (11.8)	32.9 (4.9)	31.8 (18.38)
Alkaline phosphatase (U/L)	70.7 (33)	80.5 (37)	79.9 (22)
Alanine Aminotransferase (U/L)	32.6 (10)	55.1 (10)*	79.4 (26)***§
Aspartate transaminase (U/L)	66.6 (7)	169.9 (12)***	198.2 (38)***§
Lactate dehydrogenase (U/L)	323 (77)	360 (92)	396 (109)

HDL-c high density lipoprotein cholesterol, LDL-c low density lipoprotein cholesterol

Values are mean (SD); $n=9$ per group. Comparison to control diet group: *** p < 0.001, ** p < 0.01, * p < 0.05. High-energy diet group in comparison to high-sugar diet group: § p < 0.05

(Ainslie et al. 2000; El-Haschimi et al. 2000). Therefore, adipocytokine leptin serum levels were quantified. There was no significant difference in levels of leptin measured in all three groups (Fig. 1d).

Serum parameters were analyzed in different diet groups (Table 2). Biochemical analysis revealed 1.8- and 2-fold increase in serum glucose concentration in HS and HED animals, respectively. Animals in the HED diet group

presented the highest glucose levels. Creatinine concentration increased about 2-fold in both HS and HED rats compared with the control group. Total protein and urea levels had no significant differences among the groups.

Regarding the lipid profile, serum triglyceride levels were about 2-fold higher in the HED group compared with control values. Serum levels of total cholesterol were increased about 1.5-fold in HED animals compared with control values (Table 2). Both HS and HED rats exhibited an increasing trend of LDL-c levels and a decreasing trend of HDL-c levels. ALT and AST levels were increased in HS and HED animals relative to control rates. ALT concentration increased about 1.6- and 2.4-fold in the HS and HED groups, respectively, compared with the control group. Regarding AST levels, there was a 2.5- and 3-fold increase in HS and HED rats when compared with control rats. LDH and ALP levels had no significant differences among the groups.

Liver and adipose tissue histological analysis

To evaluate the effects of diets on liver cytoarchitecture hepatic tissue sections were stained with H&E (Fig. 2a). Sections from the control group exhibited a normal lobular

pattern, with a central vein and pericentral sinusoids. In addition, they exhibit a normal appearance of the portal triads and of the hepatic cord structure; the hepatocytes appear radially arranged between the adjacent sinusoids with a regular outline. The histologic analysis of hepatic sections of the HS and HED groups revealed alterations in hepatic cord architecture, with dilated sinusoids and the hepatocytes displayed a morphological feature of an accumulation of intracytoplasmic lipid droplets (Fig. 2a). In addition, HS and HED tissue sections displayed infiltration of inflammatory cells and few cell shrinkages with an irregular outline, consistent with the presence of apoptotic hepatocytes, with lobular inflammation more marked in the HED group livers (Fig. 2a).

Sections stained with Masson trichrome had a normal collagen distribution in the liver parenchyma of control rats (Fig. 2a). Collagen presents perivascular localization with scarce perisinusoidal deposition. However, liver tissue sections of HS and HED animals revealed an accumulation of collagen fibrils with a perivascular and perisinusoidal localization (see high magnification, Fig. 2a). To evaluate liver fibrosis, the Sirius Red-stained collagenous fibrotic areas (Fig. 2b) were measured. There was an increase of the

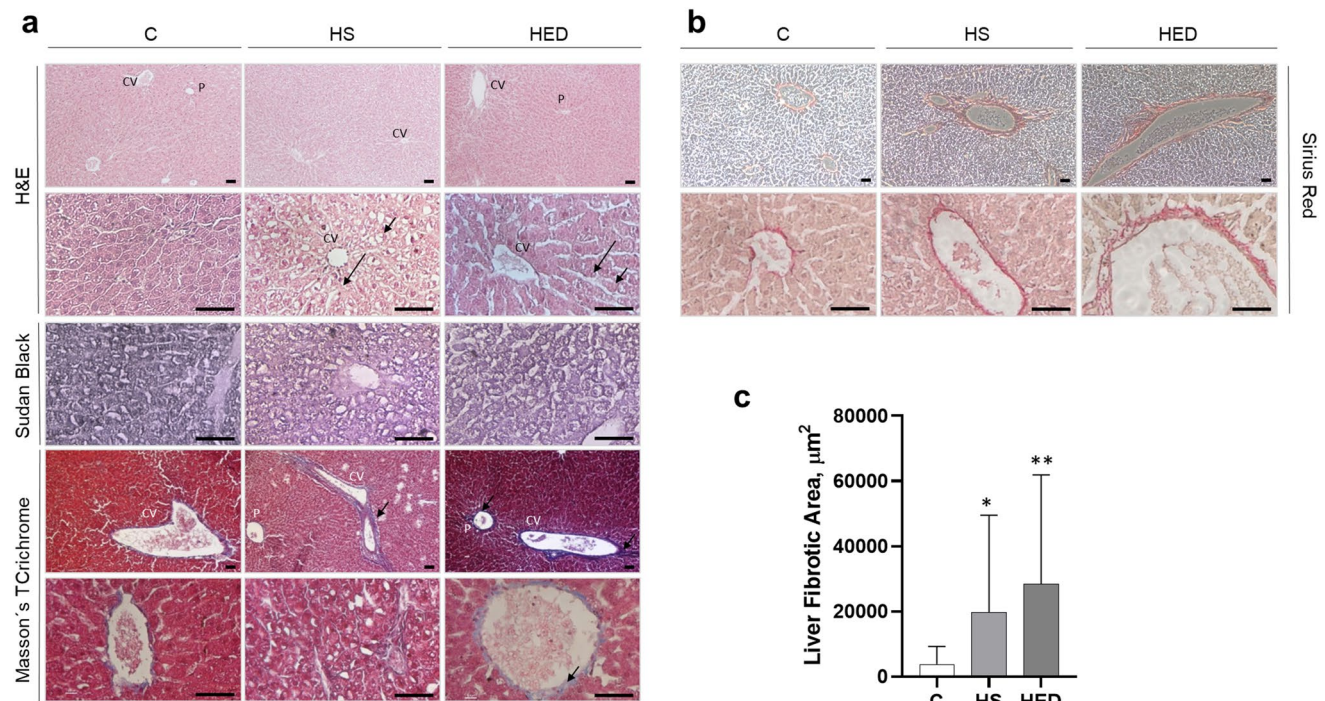
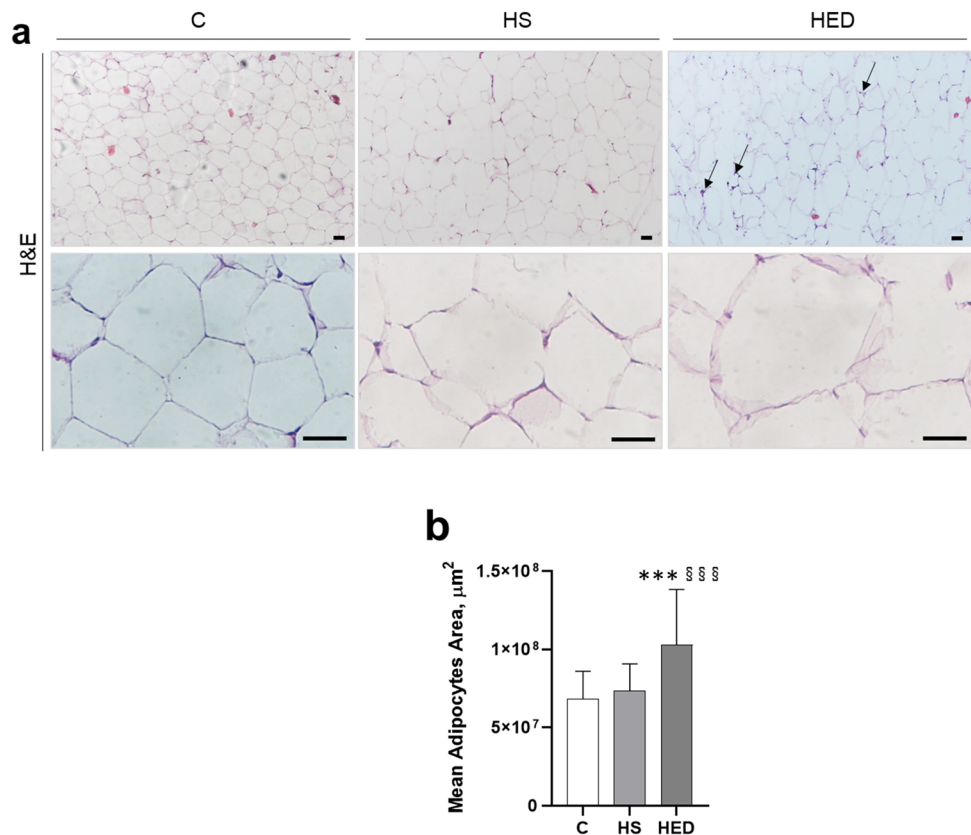


Fig. 2 Effects of diet on liver general structure and fibrosis. **a** Representative photomicrographs of liver sections stained with H&E, Sudan Black, and Masson trichrome for qualitative evaluation. Lipid droplets (short arrows), lobular inflammation, and vacuolization (long arrows) of hepatocytes were observed. Perivascular and sinusoidal (arrows) fibrosis was observed in sections stained with Masson trichrome. **b** Representative photomicrographs of sections stained with

Sirius Red used for quantification of hepatic fibrosis. **c** Graphic representation of the mean liver fibrotic area from control, HS, and HED groups ($n=9$ per group). Scale bar length is 50 μm . Data is expressed as mean $\pm$ SD; ** $p < 0.01$ vs C, * $p < 0.05$ vs C group. C control diet group, CV centrilobular vein, H hepatocytes, HED high-energy diet group, HS high-sugar diet group, P portal area

Fig. 3 Effects on diet on adipose tissue morphology. **a** Representative photomicrograph of H&E staining of adipose tissue in C, HS, and HED diet groups. The arrows point to discrete inflammatory infiltrate in adipose tissue of the HED group. In HS and HED, at higher magnification, the adipocytes show irregular shape. **b** Mean values $\pm$ (SD) of adipocytes area in adipose tissue sections of different diet groups. Scale bar length is 50 μ m. *** p < 0.001 vs C, §§§ p < 0.001 HS vs HED. C control diet group, HED high-energy diet group, HS high-sugar diet group



red-stained area in liver sections of the HS and HED groups in comparison to the control group (Fig. 2c) and the highest fibrotic area was found in the HED group.

The histological evaluation of the adipose tissue (Fig. 3a) revealed a normal histology in control animals, with spherical shape adipocytes, surrounded by an extracellular matrix with a typical regular arrangement. Remodeling of the extracellular matrix and adipocytes with variable sizes were seen in HS and HED tissue sections (Fig. 3a), showing fewer small adipocytes and an increasing number of hypertrophic adipocytes in the HED group. Quantitative data showed that the mean area of the adipocytes increased in the HED group compared with the control group (p < 0.05), but there were no differences in the mean areas of adipocytes between the HS group and other diet groups (Fig. 3b).

Expression pattern of TLR4, COX-2, and E-selectin in liver

The expression of TLR4 in liver tissue sections was distinctly different between the control group and HS and HED groups; we did not detect positive staining in the control group (Fig. 4a). Considering the semiquantitative data, the percentage of TLR4-positive cases per tissue liver sample (Fig. 5), the score value was higher in the HED group ($90\% \pm 2.62$) compared with the HS group

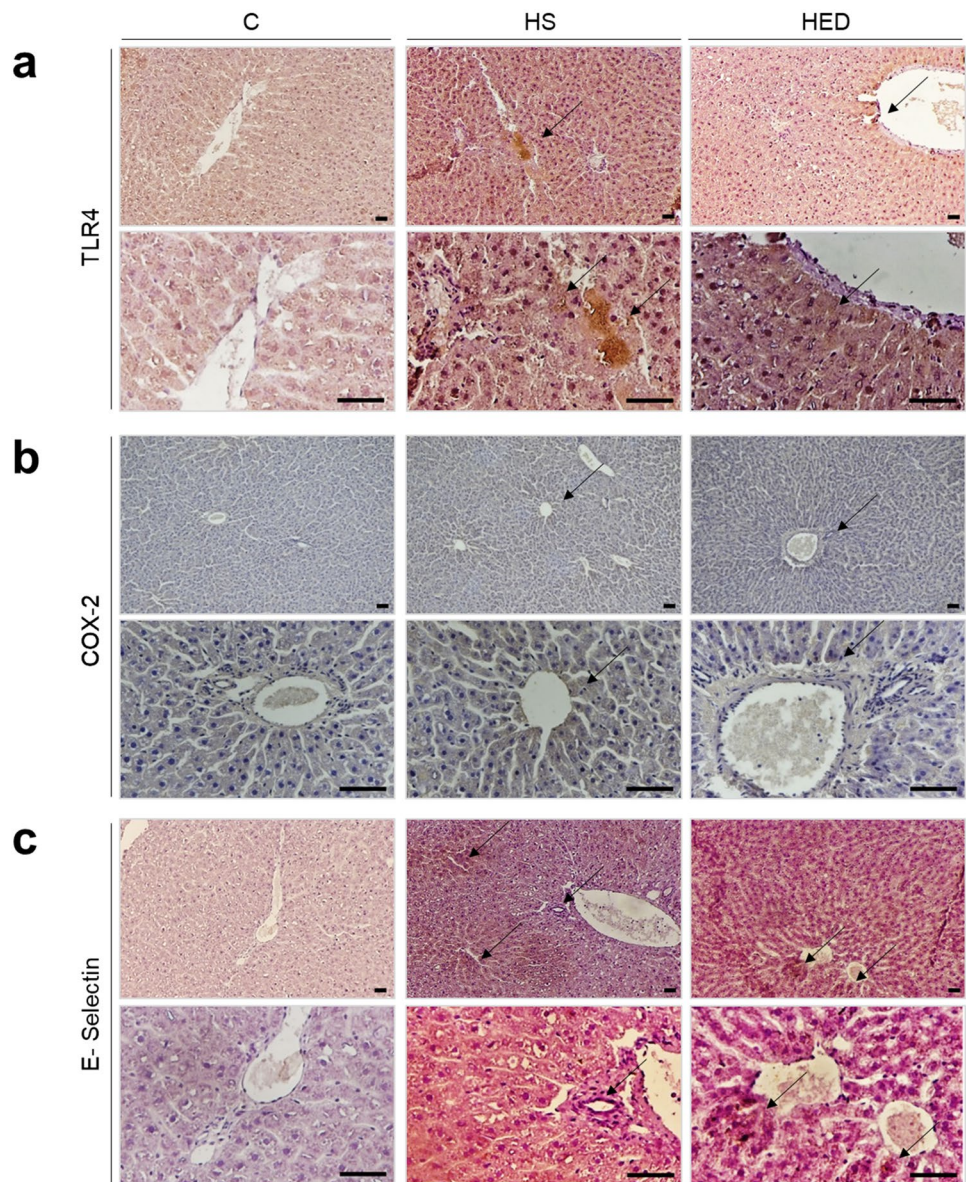
($72\% \pm 3.28$). Qualitative analysis of the COX-2 expression (Fig. 4b) revealed positivity in the hepatocytes of tissue sections from the HS and HED groups, showing similar expression pattern. The score value for COX-2 immunoexpression was $54\% \pm 2.27$ in tissue samples of the HS group and $72\% \pm 2.01$ in the HED group (Fig. 5).

E-selectin immunoexpression was observed mainly in the vascular endothelium of the hepatic tissue sections from the HS and HED groups (Fig. 4c), displaying a similar distribution, while immunoreactivity was not detected in the liver from the control group. The score for E-selectin expression was about $34\% \pm 1.50$ in the HS group and $64\% \pm 1.35$ in the HED group (Fig. 5).

Liver oxidative stress evaluation

MDA is a product of lipid peroxidation and therefore an indicator of oxidation. Figure 6 shows that there was a significantly higher content of MDA in liver samples of HS and HED rats compared with the control group (p < 0.05). The higher MDA content was determined in HED animals, but no differences are observed between HS and HED groups.

Fig. 4 Immunohistochemical expression pattern of TLR4, COX-2, and E-selectin in the liver of different diet groups. Absent immunoreaction for TLR4 (**a**), COX-2 (**b**), and E-selectin (**c**) in the control group. **a** Positive expression of TLR4 in liver sections from HS and HED diet groups. **b** COX-2 immunopositive staining of liver sections of the HS and HED diet groups. **c** Positive expression of E-selectin was observed in liver from the HS and HED groups. Arrows show the positive expression in the HS and HED groups. Scale bar length is 50 μ m. *C* control diet group, *HED* high-energy diet group, *HS* high-sugar diet group



Discussion

Clinical and experimental studies have reported that exposure to unhealthy diets during early life contributes to the development of metabolic disorders in adulthood (Lee and Yoon 2018; Weihrauch-Blüher et al. 2019; De Marco et al. 2021). Poor nutritional diets impair metabolic programming that, ultimately, may lead to metabolic syndrome (Duque-Guimarães and Ozanne 2013). In the present study, we evaluated the effects induced by the early consumption of high-sugar and high-energy diets on the liver and adipose tissue structure in male rats, addressing the contrasting effects of both unhealthy diets on metabolic function, local inflammation, and oxidative stress.

Biochemical data revealed that glucose concentrations increased in the HS and HED groups compared to control values. Whilst the HS diet rats had ad libitum access to sucrose solution with high concentration, the HED group animals had additional sucrose content from processed food consumption. Therefore, the high glucose levels exhibited by HED rats can be partially attributed to the overconsumption of added sugars, which supports previous findings (Ritze et al. 2014). Total protein serum levels showed no significant increase between groups, revealing that the general nutritional state regarding protein levels did not reflect the unhealthy eating habits. The creatinine concentration is often used to assess the level of renal function in acute and chronic conditions (Schwartz et al. 1987), suggesting that the increase of creatinine levels observed in HS and HED

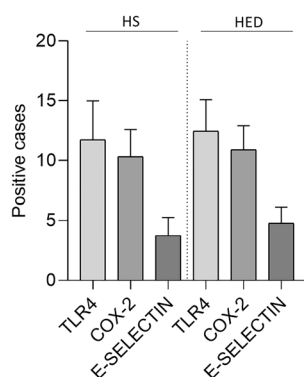
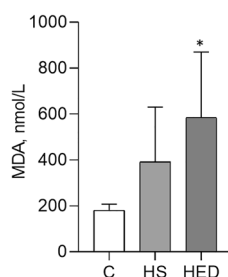


Fig. 5 Immunoreexpression score for TLR4, COX-2, and E-selectin in the liver of HS- and HED-treated animals. Data expressed as percentage of positive cases per tissue liver samples ($n=9$ per group)

Fig. 6 Lipid peroxidation products in the liver of different diet groups. Values of MDA content (nmol/L) were significantly higher in HED compared to control group ($n=9$ per group). C control diet group, HED high-energy diet group, HS high-sugar diet group. $*p < 0.05$ vs C



rats might be related to kidney dysfunction. On the other hand, we found no changes in urea levels in HS and HED animals as was expected. Indeed, it is documented that the consumption of fructose-sweetened beverage and high-fat/high-glucose diet can decrease the urea cycle in adult rats (Cox et al. 2012; Moreno-Fernández et al. 2018).

The histological and biochemical features displayed by HS and HED animals had patterns that “overlap” the criteria of NAFLD, namely: significantly increased triglycerides and total cholesterol serum levels, increased ALT and AST (Maersk et al. 2012; Peng et al. 2021). High circulating levels of ALT and AST are considered as biomarkers of liver injury, emphasizing the hepatocellular pathological alterations (Angulo 2002; Yang et al. 2014). LDH is considered a marker of tissue damage (Angulo 2002) and the lactate production is dependent on glucose availability (Digirolamo et al. 1992; Sabater et al. 2014; Arriarán et al. 2015). Although there is an increase in serum glucose levels, that does not translate into an increase in LDH levels in our experimental model. It is well established that metabolic syndrome and obesity are main drivers of NAFLD (Kim et al. 2018; Panasevich et al. 2018; Godoy-Matos et al. 2020). However, it is also known that NAFLD has a stronger positive correlation with abdominal obesity than with body mass index (Poorten et al. 2008). Corroborating these previous finding, the histopathological alterations that

we observed in the liver cannot be inferred from the differences in the total body weight but was related to an increase of abdominal adipose tissue accumulation.

Although hepatomegaly is a physical finding in most patients with NASH (Angulo 2002), we found no effect of unhealthy diets on the liver weight compared with the control group. Nevertheless, there was an increase in the adipose tissue, namely in the perigonadal and abdominal adipose tissue weight, in both HS and HED groups, revealing that both unhealthy diets may have different metabolic effects, even without a significant increase in liver weight, in agreement with previous findings (la Fleur et al. 2014). It has been reported that high-caloric diets may not induce an increase in total body weight in the juvenile period (Pini et al. 2017), suggesting that the increase in adiposity is not always accompanied by total weight gain, as our data showed. This extrapolation is supported by the literature that defines it as a condition of “obese sarcopenia” (Kalinkovich and Livshits 2017). Furthermore, sarcopenia was found to increase the risk of metabolic syndrome in non-obese people and it includes muscle loss and dysfunction, inducing contractile impairment and metabolic alterations, affecting immune and inflammatory responses (Byeon et al. 2015; Kalinkovich and Livshits 2017).

Exacerbated deposition of adipose tissue can alter the endocrine function (Ainslie et al. 2000; Xiao et al. 2020). HS and HED animals exhibited an increase of adipose tissue weight, so an elevation of leptin circulating levels was expected, since leptin is secreted mainly by adipocytes and in proportion to adipose mass (Xiao et al. 2020). However, we found no significant differences in the adipocytokine levels despite the higher adiposity index in HS and HED groups. It is known that leptin has metabolic effects in peripheral tissues, including stimulation of fatty acid oxidation and alterations in leptin signaling, being associated with NASH (Angulo 2002; Könnner and Brüning 2012). In addition, it has been suggested that a high-fat diet may contribute to leptin resistance due to a defect in peripheral leptin ability to activate hypothalamic areas (Ainslie et al. 2000; El-Haschimi et al. 2000). Leptin can act via activation of two proteins that inhibit the Na^+/K^+ -ATPase, suggesting a relationship between the levels of leptin and the decreased expression levels of the kidney Na^+/K^+ -ATPase (Briffa et al. 2015). Moreover, plasma leptin concentrations can be influenced not only by total adiposity but may be also modulated by starvation-hormonal factors, such as gonadal steroids, insulin, and glucocorticoids (Rayner and Trayhurn 2001). The interaction and influence of different metabolic pathways and hormones are possible explanations that deserve further investigation. Despite the higher adiposity index exhibited by HS and HED groups, no significant differences in leptin levels were observed. Previous studies have also found diverging results in rodents fed with a

high-fat or combined high-sugar diets (Ainslie et al. 2000; Cha et al. 2000; Francisqueti et al. 2017; Moreno-Fernández et al. 2018). In addition, high-fat diets disrupted the daily pattern of leptin levels with significant changes in diurnal leptin peaks (Cha et al. 2000).

Hepatic fibrosis has been associated with NASH development, which is characterized by increased deposition of collagen fibrils in the blood vessel walls and ballooning degeneration of hepatocytes (Peverill et al. 2014). These alterations of the normal liver architecture were also observed in both HS and HED rats, being exacerbated in HED.

Pathological lipid accumulation in the liver, mostly triacylglycerol and altered triglyceride secretion, can lead to steatosis (Gluchowski et al. 2017). Our results showed an accumulation of lipid droplets and sinusoidal dilatation in the liver of animals fed with HS and HED diets, suggesting that both diets may impair lipid metabolism in the liver. It is possible that both diets may cause activation of endoplasmic reticulum stress, triggering a lipogenic program in the liver and promoting insulin resistance (Özcan et al. 2004; Lee et al. 2008; Gregor and Hotamisligil 2011; Gluchowski et al. 2017). Moreover, we showed that HED diet induced marked hepatocellular injury, in agreement with a previous report that demonstrated the effects of diet composition (lipids and carbohydrates content) on liver lipid accumulation and inflammation (Da Silva-Santi et al. 2016).

Our data revealed an increase of positive immunostaining for TLR4 and COX-2 in hepatocytes of the HS and HED rats, whereas the increase of positive E-selectin immunorexpression was detected in the liver vascular endothelium. In addition, TLR4, COX-2, and E-selectin immunorexpression revealed a high positivity score in the liver of HED rats. TLR-4 is critical in glucose and lipid metabolism and its expression can result in systemic and local inflammation, which include an increased production of proinflammatory cytokines through the activation of the transcription factor NF- κ B, leading to insulin sensitivity (Könner and Brüning 2012; Francisqueti et al. 2017; Porras et al. 2017). Expression of COX-2 is typically induced in response to cell stressors, such as NF- κ B stimulation, being overexpressed in hepatocytes after liver injury and in liver pathology (Martín-Sanz et al. 2010; Francés et al. 2015; Motiño et al. 2019). However, transgenic models studies showed that COX-2 expression may exert distinct liver effects on normal physiology or after proinflammatory stimuli, including a protective role or enhanced liver injury (Martín-Sanz et al. 2010). For example, after acute liver injury, hepatocyte COX-2 induction had protective effects involving signaling pathways that attenuate inflammation and OS (Motiño et al. 2019). Moreover, in an adult transgenic model fed a high-fat diet, expression of COX-2 in hepatocytes induced a decrease of hepatic steatosis, inflammation, and insulin resistance (Francés et al.

2015). Our results further support these findings by showing that young male Wistar rats submitted to long-term HS and HED diets exhibited an increase of hepatocyte COX-2 expression. These findings allow us to propose that COX-2 expression might have a counterbalancing effect, preventing excessive liver lipid accumulation and a worsening of serum lipid profile induced by early unhealthy eating habits.

E-selectin is a vascular cell adhesion molecule, known to be upregulated under inflammatory conditions in experimental animals and humans studies, has a key role in the development of steatosis and NASH (Rodrigues et al. 2021). Corroborating previous reports (Simons et al. 2020; Rodrigues et al. 2021), we showed that the pattern of E-selectin expression was on vascular endothelium of the liver from HS- and HED-fed animals, being a marker indicative of inflamed blood vessels (Simons et al. 2020). E-selectin is specifically expressed on cytokine-activated endothelial cells of liver in response to systemic inflammation or liver injury, leading to leukocyte extravasation from the vasculature as a part of the inflammatory response (Rodrigues et al. 2021). A NASH genetic animal model was used to show that Western diet feeding of adult mice induced an increase of hepatic E-selectin expression, which was related to inflammatory stage (Simons et al. 2020). In addition, an increase of hepatic proinflammatory signal and E-selectin can induce the activation of TLR4, triggering OS and the upregulation of profibrogenic signal, events that lead to liver fibrosis onset (Ignat et al. 2020). Our results support previous reports (Simons et al. 2020; Rodrigues et al. 2021) by showing that normal rats fed both HS and HED diets induced an increase of TLR4 expression, increase of ALT levels and hepatic E-selectin expression, alongside an increase of hepatic lipid peroxidation.

The link between OS and inflammation seems to share the same TLR signaling pathways. In addition, OS activates inflammatory kinases, triggering vascular dysfunction in response to metabolic changes and vice versa, changes that can be aggravated leading to metabolic diseases such as diabetes and NAFLD (Könner and Brüning 2012; Porras et al. 2017; Silva et al. 2018). The adipose tissue is also particularly susceptible to OS, as it induces adipocyte proliferation and differentiation, stimulates deposition of adipose tissue, and drives inflammation that may perturb insulin signaling (Johnson et al. 2016; Kelly et al. 2016). In the visceral adipose tissue of HED animals, we showed adipocyte hypertrophy along with an abnormal structure. Growth and expansion of adipocytes may not be accompanied by a flexible extracellular matrix and a concomitant adaptation of blood flow (Lee et al. 2010; Johnson et al. 2016). Although we saw differences in the mean adipocyte area of HS animals compared to the control group, we observed some abnormal changes of adipose tissue cytoarchitecture, suggesting that pathological changes may occur within the extracellular

matrix. Hypertrophic adipocytes may cause per se insulin resistance because they express more beta-3 adrenergic receptors, which promote monocytes diapedesis, contributing to an inflammatory state and leading to augmented lipolysis (Fernández-Sánchez et al. 2011; Kim et al. 2015).

The present study revealed the long-lasting negative effects of the consumption of HS and HED diets on liver, adipose tissue, and metabolic-related parameters, with HED leading to a more pronounced adiposity and distinct hepatic phenotype characterized by increased inflammation and oxidative stress. We identified increased adiposity index with the consumption of unhealthy diet, although there was no variation in body weight gained. Therefore, it is crucial to establish more accurate measurements to access excessive adiposity, as well as to identify the metabolic impairment early to prevent the development obesity-related disease, including NAFLD.

Author contributions SN contributed with laboratory work, data analysis and interpretation, preparation of the figures and the manuscript. BMF contributed with data analysis and interpretation and revising the manuscript. RS contributed with data analysis and interpretation and revising the manuscript. SL contributed to the conception and preparation of the study, data analysis and interpretation, and revising the manuscript. FG and LCM contributed with laboratory work and data analysis. AC contributed to the conception of the experimental model and revising the manuscript. SIS contributed with the conception and preparation of the study, laboratory work, data analysis and interpretation and revising the manuscript. All authors approved the final version of the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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3.2

Unhealthy diets induced distinct and regional effects on intestinal inflammatory signaling pathways and long-lasting metabolic dysfunction in rats

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Article

Unhealthy Diets Induce Distinct and Regional Effects on Intestinal Inflammatory Signalling Pathways and Long-Lasting Metabolic Dysfunction in Rats

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Abstract: The intestinal epithelium is a principal site for environmental agents' detection. Several inflammation- and stress-related signalling pathways have been identified as key players in these processes. However, it is still unclear how the chronic intake of inadequate nutrients triggers inflammatory signalling pathways in different intestinal regions. We aimed to evaluate the impact of unhealthy dietary patterns, starting at a younger age, and the association with metabolic dysfunction, intestinal inflammatory response, and obesity in adulthood. A rat model was used to evaluate the effects of the consumption of sugary beverages (HSD) and a Western diet (WD), composed of ultra-processed foods. Both diets showed a positive correlation with adiposity index, but a positive correlation was found between the HSD diet and the levels of blood glucose and triglycerides, whereas the WD diet correlated positively with triglyceride levels. Moreover, a distinct inflammatory response was associated with either the WD or HSD diets. The WD induced an increase in *TLR2*, *TLR4*, and nuclear factor-kappa B (*NF-κB*) intestinal gene expression, with higher levels in the colon and overexpression of the inducible nitric oxide synthase. In turn, the HSD diet induced activation of the *TLR2*-mediated *NF-κB* signalling pathway in the small intestine. Altogether, these findings support the concept that early intake of unhealthy foods and nutrients are a main exogenous signal for disturbances of intestinal immune mechanisms and in a region-specific manner, ultimately leading to obesity-related disorders in later life.

Keywords: western diet; high-sugar diet; toll-like receptors; iNOS; intestinal inflammation; long-lasting metabolic effects

1. Introduction

Consumption of ultra-processed foods and sugary beverages are some of the main contributors to the development of obesity and its related comorbidities [1–3]. Although

obesity is defined as abnormal body fat accumulation, the biological processes involved are complex and go beyond excessive energy intake. It is recognized that the activation of the immune system and chronic low-grade inflammation are mechanisms involved in obesity-related metabolic disease [4,5]. The disruption of the microbiota–host interactions for diet early in life may account for the deviant programming of immunity and metabolic disorders later in life [6–9]. Extensive research has suggested that the link between diet and obesity-induced inflammation seems to be closely related with an impaired intestinal barrier function [10–15], which can play a crucial role in the disruption of energy homeostasis. However, how macronutrients' intake affects metabolic and immune systems is still uncertain. Moreover, the mechanisms underlying the link between obesity-related diets and metabolic inflammation remain poorly understood and may vary depending on tissue types and location.

The intestinal epithelium represents the largest interface for the occurrence of an intricate microbiota–host crosstalk, which is strongly influenced by luminal environment and internal signals [16–18]. Among the external cues, both dietary macronutrients and microbiota compounds interact with the intestinal barrier to modulate the signalling pathways controlling physiologic functions, such as digestion, absorption, immunity, and metabolism [17]. The activation of defence mechanisms requires specialized receptors, collectively referred to as pattern recognition receptors, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [16,19,20]. Activation of both receptors induces nuclear transcription factor-kappa B (NF- κ B), a signalling pathway that regulates immune functions and gene expression related with inflammation and metabolism [21–24]. Moreover, failure of these regulatory pathways is a trait shared by several intestinal inflammatory diseases, which is also implicated in the development of obesity- and diabetes-related metabolic dysfunction [5].

Mice fed high-saturated fat showed changes in insulin sensitivity and adipose tissue inflammation, associated with the upregulation of *TLR4* and *TLR2* signalling [25]. In intestinal tissue, high-fat diets seem to specifically increase *TLR4* expression [24], but there is less information about the impact of obesogenic diets on the intestinal *TLR2* expression. Throughout the intestinal epithelium, the TLRs are expressed in both immune and non-immune cells, including in enteric neurons, reinforcing their crucial role in intestinal homeostasis [16,20,26–28]. In addition, intestinal *TLR2* and *TLR4* expression shows region-specific patterns [21,26,29]. Furthermore, it was shown that high-fat feeding [30] and an intestinal inflammatory state induced the upregulation of intestinal inducible nitric oxide synthase (iNOS) and, hence, NO production, suggesting it to be a crucial immunomodulator of the intestinal motility [31].

In the context of the immune cell sensors involved in metabolic inflammation, there is increasing interest in the role of the NLR family, specifically the *NLRP3* inflammasome [32]. High-fat feeding of *NLRP3*-deficient mice exhibited a favourable effect on insulin sensitivity and in adipose tissue inflammation related with obesity [32,33]. Moreover, *NLRP3* is induced not only by microbial components, TLR ligands, and the transcription factor NF- κ B, but also by fatty acids [22,32,34,35], suggesting its potential regulatory role in metabolic inflammation. However, the contribution of the *NLRP3* inflammasome for the intestinal inflammatory response in diet-induced obese mice models has yet to be elucidated.

Western diets (WDs), typically with low dietary fibre, can shape microbiota composition [14,36,37]. In turn, mucin glycans are used as nutrient sources for bacteria, resulting in an erosion of the mucus barrier, an increase in intestinal permeability, and a decrease in beneficial microbiota-derived metabolites, such as short-chain fatty acids (SCFAs) [18,36,38]. Furthermore, the mucus layer overlaying the epithelium is produced by goblet cells under a direct immune regulation, responding to variable stimuli in a region-dependent manner [39]. Although not fully explored, current evidence has established a causative association between high saturated-fat intake and perturbation of gut microbiota, leading to intestinal permeability, proinflammatory responses, and dysmotility [12,23,27,40–43]. For example, in mice models, high-fat feeding induced an upregulation of jejunal inflam-

matory gene transcripts associated with impaired glucose homeostasis, without changing adiposity [40]. Meanwhile, high-fat plus high-sugar feeding induced a higher body weight gain, accompanied by hyperproliferation of intestinal stem cells and changes of enterocytes in a regional-specific manner [44]. Furthermore, distinct effects of a high-fat diet on the intestinal barrier function were also shown in other studies, ranging from marginal in the ileum and colon to severe in other segments of the small intestine [42,45].

It has been shown that intake of added sugars early in life affected intestinal microbiota in rats, and this effect was obesity-independent [46]. Meanwhile, mice on a high-sugar diet exhibited glucose homeostasis impairment and obesity [47], disruption of gut epithelium integrity, and systemic inflammatory response [48]. However, research on the effects of early high-sugar diets on intestinal inflammatory response and its metabolic outcomes is limited.

Whether immune regulation is distinctly affected by the consumption of WD and high-sugar diet (HSD) is still unknown. Therefore, the main goal of this study was to investigate how early unhealthy dietary patterns influence intestinal inflammatory responses and their association with metabolic outcomes in later life, by comparing two specific diet patterns in the development of obesity and its impact on intestinal inflammation.

2. Results

2.1. Effects of Energy and Macronutrient Intake on Anthropometric Parameters

Data analysis confirmed possible obesogenic effects induced by HSD and WD (details in Section 4) in rats under these dietary constraints from an early age until adulthood. Total energy intake per day consumed by WD-fed rats was higher than the one of rats fed with a chow diet (CD) or HSD (Figure 1a). Regarding diet composition, WD-fed rats had higher energy from fat, particularly high saturated fat intake, and consumed a lower content of dietary fibres compared to the other feeding groups (Figure 1b,c,f). The amount of simple sugar intake in WD-fed rats was higher only when compared to the CD group (Figure 1e). Although HSD-fed rats had free access to sucrose solution instead of water, like CD-fed rats, this group presented a lower total energy intake, caused by a reduction in chow diet intake, which was concomitant with a reduced cellular nutrient supply, including fibre intake (Figure 1d–f). Moreover, protein intake was greater in CD-fed rats than in those fed with a HSD or WD (Supplemental Table S1).

Lifelong dietary intervention induced significant variation on body weight, visceral adipose tissue weight, and adiposity index, while there was no significant difference in body weight gain (Supplemental Figure S1). The mean body weight of WD-fed rats was about 5% and 13% higher than that of CD- and HSD-fed rats, respectively. Nonetheless, WD feeding induced a sharp increase in adipose tissue weight and rats displayed a greater adiposity index compared with HSD- and CD-fed rats (Supplemental Figure S1c,d). Interestingly, HSD-fed rats had the lowest body weight values, but their adipose tissue was about 44% heavier than that of CD-fed rats and 33% lighter than that of WD-fed rats.

To further investigate the effects of macronutrients on body composition, we performed a Pearson correlation analysis and explored the association between the intake of each dietary component and obesity index (Figure 2), conditioned on body weight gain. We found that all analysed dietary components (Supplemental Table S2) were correlated with the adiposity index. Among these, we showed that saturated fat (g) and sugar (g) intakes were positively correlated with the adiposity index, whereas protein and dietary fibre (g) intakes had a negative correlation (Figure 2d,e).

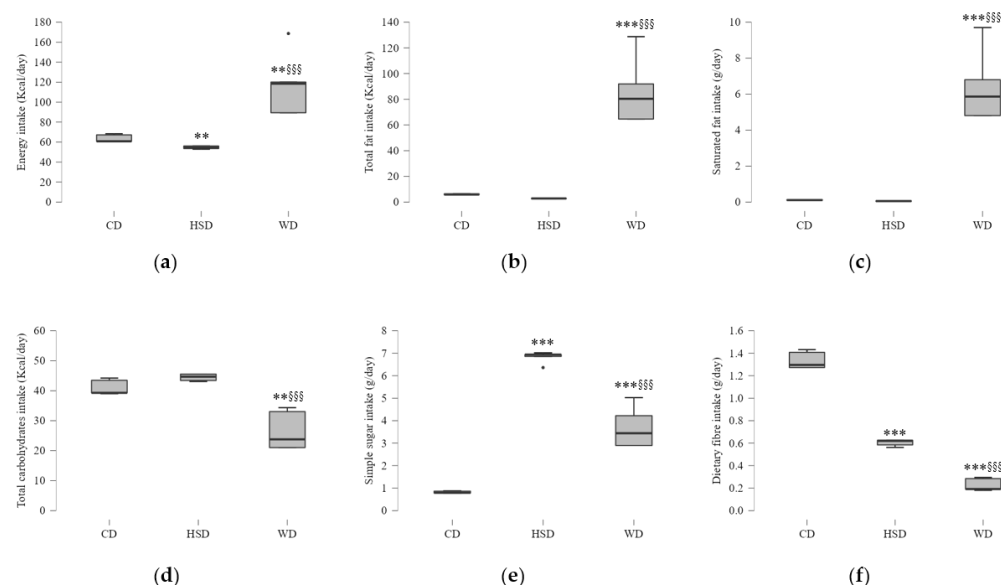


Figure 1. Graphic representation of the daily intake of energy and nutrients from chow-fed (CD), high-sugar diet (HSD), and Western diet (WD) groups. (a) Total energy intake from solid food and drink, although CD rats had access to water. (b) Energy from total fat intake. (c) Saturated fat intake (g/day). (d) Energy from total carbohydrate intake (e). Simple sugar intake (g/day). (f) Dietary fibre intake (g/day). Mean values $\pm$ standard deviation (SD) are plotted. Symbols * and § indicate significant differences (one-way ANOVA) compared to CD-fed group: ** $p < 0.01$, *** $p < 0.001$; HSD-fed group: \$\$\$ $p < 0.001$ ($n = 9$ rats per group).

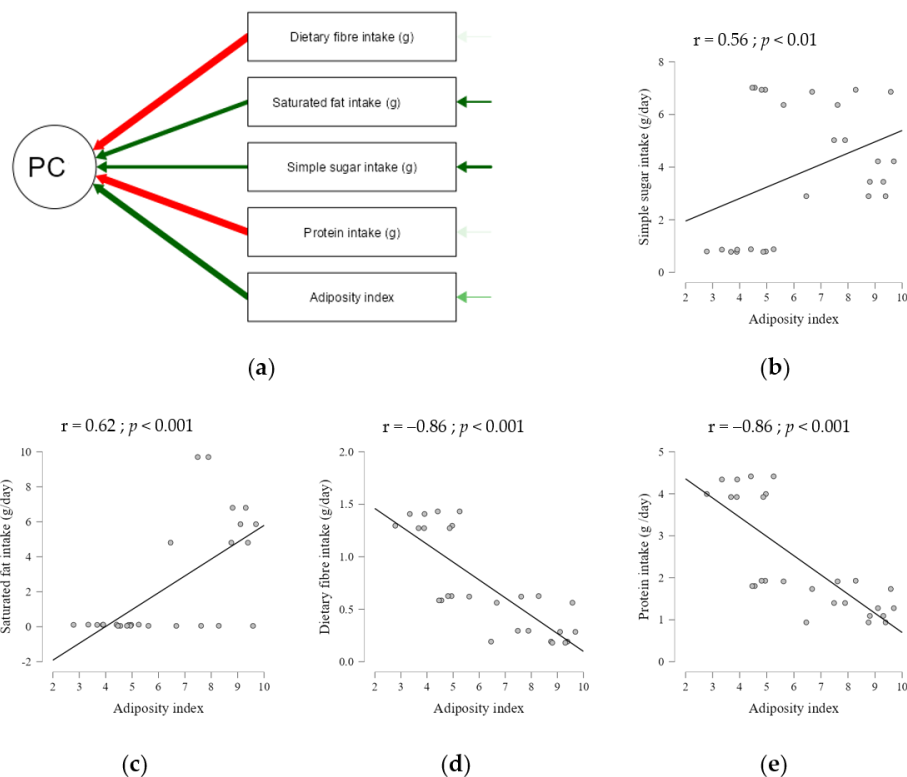


Figure 2. Correlation between nutrients intake and adiposity index. (a) Path diagram of principal component (PC) used in Pearson correlation analysis. (b) Simple sugar and (c) saturated fat intakes were positively correlated with adiposity. (d) Dietary fibre and (e) protein intakes were negatively correlated with adiposity.

2.2. Dietary Composition Effects on Serum Metabolic Parameters

After showing that both dietary patterns were obesogenic, data analysis sought to evaluate the association between nutrient intake and serum metabolic parameters, namely fasting blood glucose, triglycerides, and total cholesterol levels (expressed as mmol/L). There was a significant difference in the circulating levels of fasting blood glucose between the three groups (Supplemental Figure S2a). Glucose concentration was significantly higher in HSD-fed rats (10.76 ± 2.11) and WD-fed rats (11.48 ± 3.05), compared to the CD feeding group (6.75 ± 1.44 ; $p < 0.01$). When glucose levels were compared between the HSD and WD feeding groups, we found no significant differences in serum levels. Moreover, Pearson correlation results (Supplemental Table S3) showed that only sugar intake had a positive correlation with fasting blood glucose (Figure 3a–c).

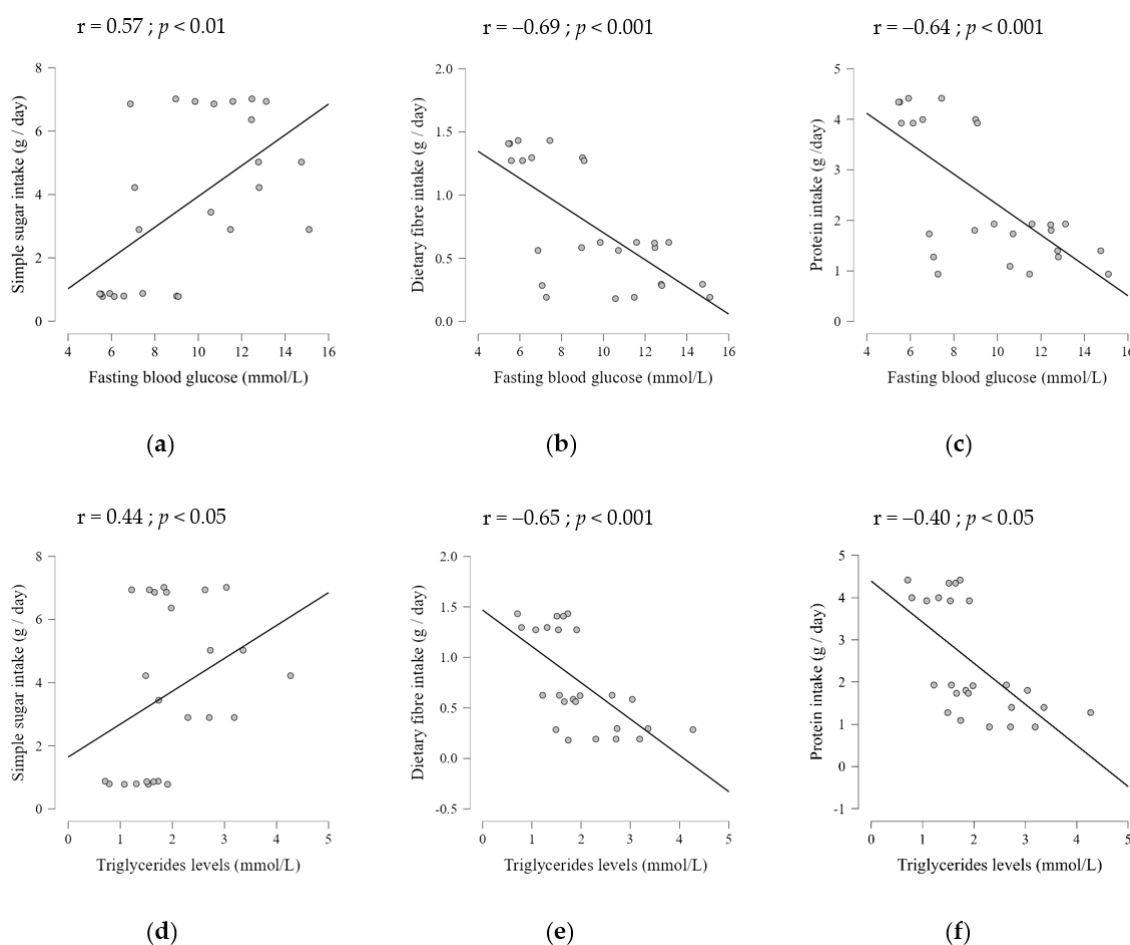


Figure 3. Correlation between nutrients intake and serum metabolic parameters. (a) Simple sugar intake had a positive correlation with fasting blood glucose, while (b) dietary fibre and (c) protein intakes had a negative correlation. (d) Simple sugar intake has positively correlated with triglycerides levels. A negative correlation was found between (e) dietary fibre and (f) protein intakes with triglycerides serum levels.

Regarding the blood lipid profile, there was a significant effect of diet on triglyceride and total cholesterol serum levels (Supplemental Figure S2b,c). No significant differences were found in blood lipid profile from rats fed with a WD or HSD, but these values were significantly different when compared with CD-fed rats. In line with the increased adiposity index, triglyceride and cholesterol levels were higher in WD-fed rats than in CD-fed rats ($p < 0.001$). HSD-fed rats had higher levels of triglycerides and total cholesterol than CD-fed rats ($p = 0.03$ and $p < 0.01$, respectively).

Pearson analysis showed that five dietary components were correlated with blood lipid parameters (Supplemental Table S3). Total cholesterol levels were correlated (negatively) only with complex carbohydrate intake, similar to the association found for triglyceride levels. In addition, we found that protein and fibre intake were negatively correlated with triglyceride levels, whereas the mean fat energy, saturated fat, and sugar intakes had a positive correlation (Figure 3d–f, Supplemental Table S3). Furthermore, all serum metabolic parameters were also positively correlated with adiposity (Supplemental Figure S3).

2.3. Effects of Obesogenic Diets on the Small Intestine Morphology

Rats fed with a HSD and WD had higher sucrose intake, which is quickly digested into glucose and fructose in the small intestine lumen. To assess whether these dietary patterns can induce changes on intestinal morphology, observation of the duodenal mucosa was undertaken in HE-stained tissue sections (Figure 4). CD-fed rats showed slender duodenal villi with regularly aligned epithelium, mainly composed of enterocytes interspersed with goblet cells and some scattered intraepithelial lymphocytes. Of note, dietary intervention induced changes in duodenal villi characteristics (Figure 4). HSD-fed rats exhibited a slight enlargement of the villi and enterocytes with misaligned nuclei, while vacuoles in the absorptive enterocytes were observed in the small intestine of WD-fed rats. From the tip to villi base, we observed fewer intraepithelial lymphocytes compared to CD feeding.

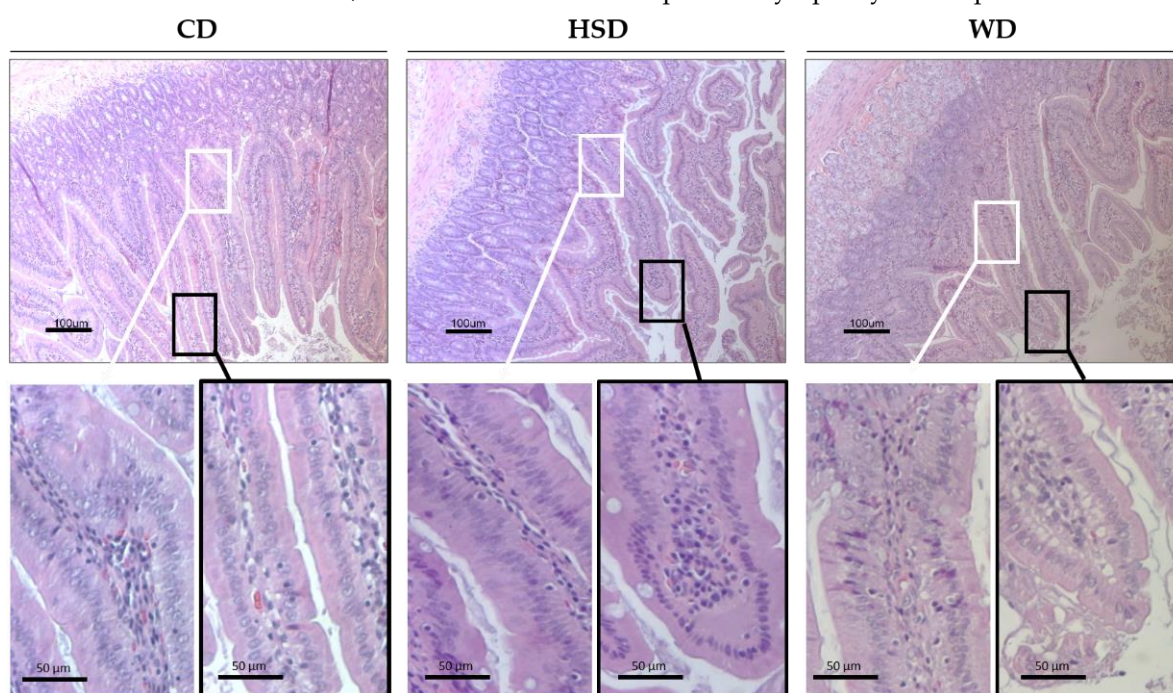


Figure 4. Dietary effects on the small intestine. Representative images of HE-stained duodenum of rats fed with chow diet (CD), high-sugar diet (HSD), and Western diet (WD). The inset shows details of the villi in small intestines, from near the villi base (white box) to the tip (black box). Villi in duodenum from CD-fed rats showed regularly aligned simple columnar epithelium and occasional intraepithelial lymphocytes. HE-staining of tissue sections from HSD-fed rats exhibited enterocytes with misaligned nuclei and slight enlargement of the villus tip. The duodenal villi of HFD-fed rats revealed vacuoles in simple columnar epithelial cells.

2.4. Distinct Effects of Obesogenic Diets on the Number of Goblet Cells

Next, we examined the HSD and WD feeding effects on the mucus-secreting cells in the same intestine segment. The acidic goblet cells that stained positively with Alcian blue in the villi epithelium were counted (Figure 5).

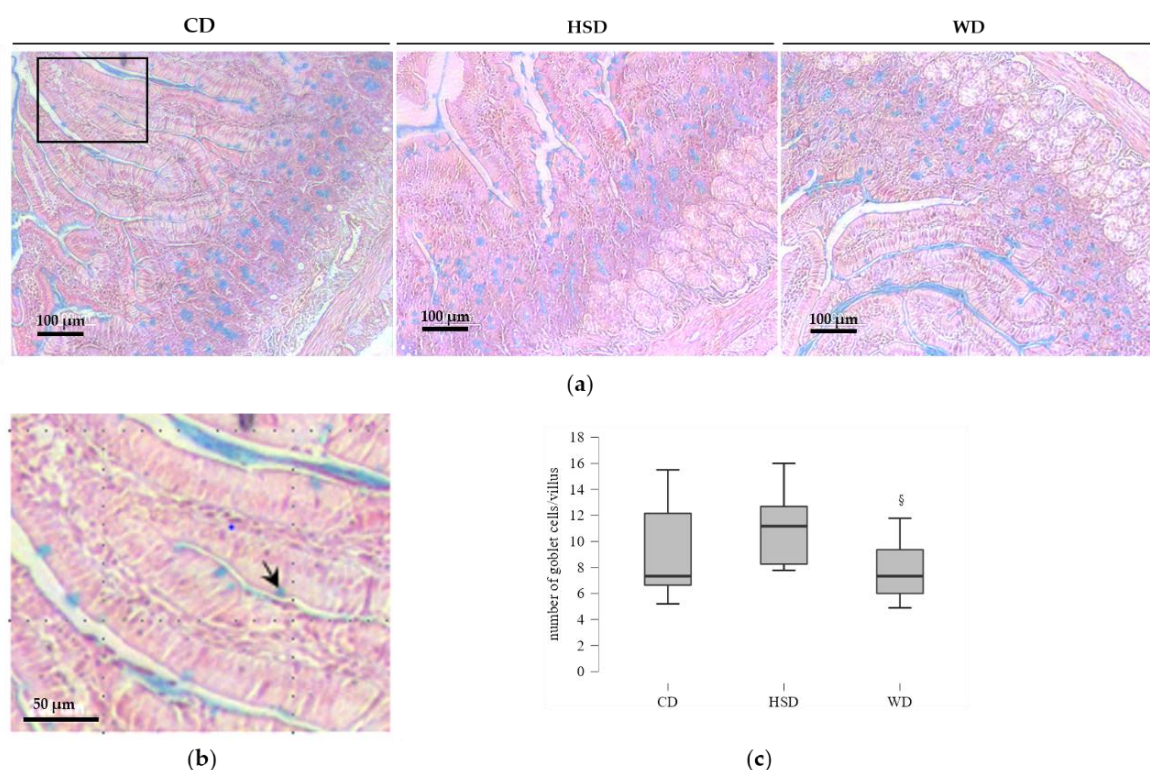


Figure 5. Dietary effects on mucus-secreting cells in the small intestinal epithelium. (a) Representative images of Alcian blue staining of transversal sections of duodenum from chow diet (CD), high-sugar diet (HSD), and Western diet (WD) groups. (b). Inset of the box showing blue stained goblet cells, which is indicated by an arrow. (c) Number of goblet cells per villus of duodenum ($n = 5–8$ villi per rat). Symbol indicates significant differences (one-way ANOVA) compared to HSD-fed group: § $p < 0.05$.

No significant difference was observed in the goblet cell number between rats fed with a HSD or CD, nor between the WD and CD feeding groups (Figure 5c). Remarkably, the mucus-positive cell number per villi in rats fed with HSD was significantly higher than that in WD-fed rats.

2.5. Distinct Effects of Obesogenic Diets on Inflammatory Signalling Pathways in the Small Intestine

Considering that the upregulation of $\text{NF-}\kappa\text{B}$ through TLR -dependent signalling triggers the production of proinflammatory cytokines, leading to the impairment of immune functions and chronic low-grade inflammation [16,19,22], we analysed the long-term effects of WD and HSD on the expression of $\text{TLR2/TLR4/NF-}\kappa\text{B}$ pathways. In addition, the expression of other key mediators for the maintenance of intestinal homeostasis, such as $i\text{NOS}$ and NLRP3 [32,34,35,49,50], were analysed.

In the jejunum, ANOVA results showed that diets induced significant variation on $\text{NF-}\kappa\text{B}$, TLR2 , TLR4 , and $i\text{NOS}$ gene expression, but there was no difference in NLRP3 expression (Figure 6a–e). The small intestine of HSD-fed rats exhibited significantly higher expression of $\text{NF-}\kappa\text{B}$ and TLR2 compared to CD-fed rats (Figure 6a,c). Although there was a slight increase in the expression of TLR4 with HSD feeding, the difference was not statistically significant compared to the levels of CD-fed rats (Figure 6b), suggesting that a HSD induced an inflammatory response in the small intestine mainly through the upregulation of the $\text{TLR2/NF-}\kappa\text{B}$ pathway. Surprisingly, in the jejunum of HSD-fed rats, a reduction in $i\text{NOS}$ expression, compared to CD-fed rats, was detected (Figure 6c,e). Regarding the WD's effects on inflammatory gene expression, the upregulation of $\text{NF-}\kappa\text{B}$,

TLR2, and *TLR4* expression was also detected in the jejunum compared to CD feeding. In addition, *iNOS* expression levels were reduced in this small intestine segment (Figure 6e).

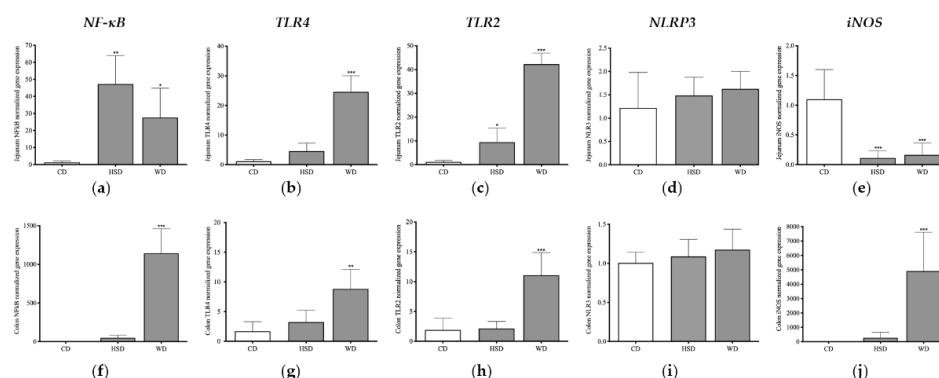


Figure 6. Graphic representation of the normalized expression levels of chow-fed diet (CD), high-sugar diet (HSD), and Western diet (WD), concerning *NF-κB*, *TLR4*, *TLR2*, *NLRP3*, and *iNOS* genes in jejunum (a–e) and colon (f–j) samples. Expression levels were normalized against the respective 18S rRNA gene expression and then against the respective controls (CD), set as 1. Mean values $\pm$ standard deviation (SD) are plotted. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

2.6. Distinct Effects of Obesogenic Diets on Inflammatory Signalling Pathways in the Large Intestine

There is evidence linking dietary fats and fibre intake with colonic microbiota composition and inflammation [23]. In this way, we further analysed the diet effects on colonic immune response. ANOVA results showed that dietary intervention also had a significant effect on *NF-κB*, *TLR2*, *TLR4*, and *iNOS* expression in the large intestine, but not on *NLRP3* expression (Figure 6f–j). Notably, there were no significant changes in the colonic expression of *NF-κB*/*TLR2* of HSD-fed rats compared to CD (Figure 6f), neither in *TLR4* expression nor in *iNOS* expression (Figure 6f–j). Meanwhile, WD intake had a significant impact on the activation of colonic-inflammation-associated pathways compared to CD intake. In addition, the upregulation of *NF-κB* expression was higher in the colon, compared with the levels detected in the jejunum ($p < 0.01$). Of note, WD intake induced a significant increase in colonic *iNOS* expression compared to CD intake and with levels in the jejunum ($p < 0.05$), where it was downregulated.

3. Discussion

Overnutrition is recognized as a main driver of the chronic metabolic inflammation associated with the development of obesity and related metabolic disorders [3,5,8,51,52]. There is the concept of dietary fat perturbation of homeostatic microbiota–host interaction, leading to intestinal immune dysregulation and barrier disruption [10,27], enabling bacterial translocation and, consequently, metabolic inflammation [13,43,53]. Both short-term and prolonged exposure to diets with high saturated fatty acids were found to induce inflammatory responses in the intestine and insulin resistance through a *TLR4*/*NF-κB*-dependent mechanism [24,54]. Other studies proposed a fatty acid-specific effect on the intestinal inflammation, through the activation of both the *TLR2* and *TLR4* signalling pathways, leading to low-grade inflammation and glucose homeostasis impairment [13,15,55].

Our results reveal that, in rats submitted to the WD, the *TLR2*/*TLR4*/*NF-κB* signalling cascade activation induced an inflammatory response in the small intestine, with a more exacerbated expression in the colon. Moreover, the colonic inflammatory response to WD was accompanied by an increased expression of *iNOS*, although its expression levels were decreased in the small intestine. In addition, we showed that high intake of saturated fat and added sugar intake by young rats were correlated with worse metabolic profiles in adulthood. It was previously reported that, in mice, diet more severely affected the intestinal barrier in the proximal small intestine than in the colon [27,42,45]. However, other previous findings showed diet-induced colonic inflammation and intestinal permeability

occurring through the induction of the *TLR4* and *iNOS* signalling pathways [54]. In addition, dietary fat induces disruption of colonic microbiota and triggers bacterial growth in the small intestine in mice [56]. Together, these findings suggest that early consumption of a WD induces disruption of regulatory signalling pathways in a region-specific manner, providing support to the causative link between ultra-processed food intake, colonic inflammation, and metabolic dysfunction.

Interestingly, we found that HSD feeding induced the activation of the *TLR2/NF-κB* pathway only in the small intestine. In line with previous studies, we observed morphological alterations in the small intestine induced by high sugar intake, including a higher number of goblet cells compared with WD feeding. The crucial role played by the *TLR2*-mediated signalling pathways in the regulation of the intestinal epithelial barrier has been recognized [16,29]. *TLR2* expression is shaped by the microbiota, and it reflects bacterial load, displaying low expression levels in the small intestine and higher ones in the proximal colon [26]. In the small intestine, microbiota shaping associated with the upregulation of *TLR2* expression leads to an increased cell turnover [21], changes in tight junction protein expression, and stimulation of mucus secretion by goblet cells [16,19]. In addition, early HSD feeding triggered an increase in adiposity in adulthood, which was correlated with unfavourable metabolic parameters. Indeed, previous studies reported the negative impact of high sugar intake on intestinal structure and function [52,57], as well as on other metabolic organs [48,58]. Therefore, it is plausible to suggest that a sustained high luminal concentration of glucose due to high sugar, per se, induces the upregulation of *TLR2* expression in the small intestine and, consequently, may lead to chronic intestinal permeability, hyperglycaemia [52,57] and, metabolic endotoxemia [8], which may contribute to the onset and progression of obesity.

We showed that only WD feeding induced the *NF-κB/TLR4/iNOS* signalling activation in the colon. Since both obesogenic diets were low-fibre, it is plausible to suggest that the deregulation of *iNOS* signalling pathway induced by saturated fat might be an internal signal to the disruption of microbiota–host interactions, leading to colonic inflammatory response and dysmotility. Increasing evidence supports the link between diet-induced obesity and intestinal motility disorders, indicating dietary fat as a main contributor to dysfunction of myenteric neurons, which leads to intestinal motility disorders [20,23,31]. Diet-induced colonic inflammation and dysmotility were reported to involve the activation of *TLR4* and *iNOS* signalling [20,23,31]. Dietary fibre intake strongly influences the microbiota–host interactions, interfering with overall metabolic function and, locally, modulating intestinal homeostasis [14,18,23,36,37]. This non-digestible carbohydrate is used by the bacteria mainly in the large intestine, producing SCFA and indole derivatives, which modulate several intestinal functions, such as barrier integrity, energy homeostasis, gut hormone production, and motility [14,23,37].

Convincing evidence links *iNOS* levels with microbiota-immune-nerve interactions, indicating the deregulation of *iNOS* signalling to play a role in colonic dysmotility [31,59]. A concept that emerged from animal models of colitis [31,59,60] linked colonic inflammation with the expansion of *Proteobacteria* [59] and increased oxidative stress [31,59], triggering the upregulation of *iNOS* expression in enteric glial and immune cells [31,60,61]. In addition, dietary fat was shown to induce colonic inflammation through the activation of *NF-κB/TLR4* signalling, leading to an increase in *iNOS* expression [23] and a subsequent increase in NO levels released by enteric glia cells in an excitation state [60]. Moreover, our previous study showed that consumption of ultra-processed foods induced the expansion of *Proteobacteria* [62], supporting the hypothesis that the diet-related *iNOS* expression in colon can be caused by disruption of microbiota-immune-nerve crosstalk, contributing to colonic dysfunction [31,60].

We found a downregulation of jejunal *iNOS* expression in rats fed with ultra-processed foods and sugar drinks. Dietary fat was previously reported to induce an increase in *iNOS* expression in the small intestine from rats [30] and mice [40]. This divergence may be explained, at least in part, by differences in animal strains and the nutrient composition

used, not only in dietary intervention timing and duration, but also in the small intestine segment analysed. Nevertheless, in *iNOS*-deficient mice, a sustained increase in the bacterial load in the ileum was shown, suggesting that *iNOS* expression might be important for inhibition of a bacterial reflux from the large intestine [49]. Since we found a decrease in *iNOS* expression in the small intestine due to WD and HSD feeding, it is possible that this effect could be the consequence of a chronic defect in the immune response, caused, in part, by dietary effects on the jejunal bacterial load. In addition, we observed fewer intraepithelial lymphocytes in the duodenal tissue sections of WD-fed rats, corroborating previous studies [63]. Alongside with *iNOS* and NO roles in intestinal barrier function [49], their role in the regulation of enteric glucagon-like peptide-1 (GLP-1) sensitivity through a *CD14/TLR4* dependent mechanism is also proposed [64]. Indeed, the expression of GLP-1 receptors was shown to be reduced in the jejunum of mice on a high fat diet but was unchanged in the colon [64]. It can be hypothesized that a sustained intestinal proinflammatory responses triggered by early dietary patterns affects immune system development, leading to intestinal function impair and deregulation of gut hormone secretion as well as causing a low-grade systemic inflammation and subsequent infiltration of immune cells in several metabolic organs [8,13,23,40–42,44].

During acute inflammatory response, the small and large intestines showed an up-regulation of *NLRP3* expression upon injury or microbial stimuli, involving the activation of *TLR4* and *TLR2* signalling [34,35]. Besides the activation by bacterial infection, this signalling pathway can be activated by saturated fatty acids [32,35], being suggested to play a role in immune dysfunction and chronic inflammation, triggering diet-induced obesity and insulin resistance [32,33]. Interestingly, we found that neither WD nor HSD feeding had influence on *NLRP3* expression in the small and large intestines. These results provide information suggesting that saturated fat intake induced neither a direct effect on the activation of intestinal *NLRP3* nor an indirect effect through the bacteria, although we cannot exclude the possibility of *NLRP3* inflammasome activation by other stimuli. The existence of an acute inflammatory response induced by high saturated fat is not expected, though a low-grade inflammation is expected. Indeed, contrarily to other metabolic tissues, the intestine possesses a tissue-resident phagocyte population that is hyporesponsive to microbial stimulation [34], suggesting that inflammasome activation might have tolerance towards non-pathogenic microbiota.

Taken together, the data indicate that the chronic consumption of ultra-processed foods induces exuberant colonic inflammatory response, contributing to metabolic consequences in adulthood, whereas the effects of sugar beverages on inflammation mechanisms were mainly observed in the small intestine. These observations support the concept that the intestinal structure and its responsive mechanisms can be disrupted by direct and indirect effects of different nutrients in a regional-specific manner.

4. Material and Methods

4.1. Animals and Experimental Diets

All procedures in this study were reviewed and approved by the Animal Care and Use Committee (ORBEA) of the Faculty of Medicine of the University of Porto (Portugal). The procedures were performed by accredited scientists (licensed users of the Federation of Laboratory Animal Science Associations—FELASA) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. Data in the current paper pertain to rats involved in a dietary manipulation experiment, some aspects of which have already been published. These previous publications have included patterns of body weight, adiposity, serum lipid profile, and serum glucose levels [65].

We previously exposed male Wistar rats, aged 4 weeks, to three dietary treatments ($n = 9$ per diet group) for 14 weeks. The number of animals per group considered previous studies in our lab and the fact that HSD group presented a statistically significant difference at “ p ” values, ranging from 0.05–0.02 and at varying 80–90% probability (levels of power) for $n = 9$ and the WD group, where the number need was lower. Throughout the study,

animals had ad libitum access to liquid and solid food, which was replaced daily. The control diet group (CD) was fed with standard chow diet (4RF21/C Mucedola, Settimo Milanese, Italy) and had access to water. The high-sugar diet group (HSD) was fed with standard chow diet and had access to 30% sucrose solution (Sigma-Aldrich Company Ltd., Madrid, Spain; 1.2 Kcal/mL). The Western diet group (WD) was fed with standard chow diet mixed with a selection of palatable human foods and had access to 15% sucrose solution (0.6 Kcal/mL). Full details on macronutrient composition of the diets are found in the supplementary material (Table S1). During the whole experimental period, rats were housed two per cage to avoid social isolation, under controlled 22–24 °C temperature and 12:12 h light/dark cycle conditions. More information about procedures and experimental design can be found in our previous paper [65]. Food intake was obtained by subtracting the final weight of the food from its initial weight, including any food pellets that had spilled into the cage.

4.2. Tissue Collection

Animals were sacrificed under deep anaesthesia, induced with sevoflurane (SevoFlo, Abbott Laboratories Ltd., Maidenhead, UK). The small and large intestines were dissected and washed in PBS to remove luminal contents. Then, ~2 cm-long sections of the duodenum were collected and fixed in 4% (*w/v*) neutral-buffered formalin solution (Sigma-Aldrich) for 24 h at room temperature. Tissue samples of jejunum and colon were quickly frozen and stored at −80 °C, until being processed for extraction of total RNA for quantitative real-time PCR analysis. Visceral adipose tissue (VAT) was weighted to assess the degree of obesity, by using the adiposity index, expressed as the ratio of VAT weight to body weight (g/100 g).

4.3. Histological Analysis and Goblet Cell Staining

Specimens of cross-section segments of duodenum were embedded in low-melt paraffin wax and cut into 4 µm thick histological sections on a microtome (Shandon™ Finesse™ 325, Thermo Scientific, Runcorn, UK). For histopathological examination, tissue sections were stained with hematoxylin and eosin (HE) (Diapath, Martinengo, Italy) and evaluated according to: widening and shortening of intestinal villi, infiltration of intraepithelial lymphocytes, and nucleus position within the absorptive cells (enterocytes) of the intestinal epithelium. The HE sections were selected for quantitative evaluation when the duodenal villi were well-oriented, displaying a surface epithelium composed mainly of enterocytes intermixed with goblet cells. Away from the regenerative zone of villus, the enterocytes were identified as columnar cells with an oval nucleus, normally occupying a uniformly basal position in the enterocyte. The differentiation between enterocytes and goblet cells was recognized by the typical pear shape of goblet cells. The Alcian blue staining (at pH 2.5) counterstained with hematoxylin was used to quantify goblet cells that contained acid non-sulphated mucins. Goblet cells were counted in both sides of the villi from base towards the tip of the villi, excluding the stem cell zone. Only villi that were cut longitudinally and with defined limits were used for quantification of goblet cells. The number of goblet cells in villi per tissue section was counted, and the average number of goblet cells per villus was estimated on 5–8 villi per animal (*n* = 9/group). This histological assessment was performed in a blind manner to the group by one of the authors. Slides were observed under phase contrast microscopy, using 100× and 600× magnifications (Eclipse TE2000-U microscope, Nikon, Melville, NY, USA), coupled to a DXM1200F digital camera and controlled by Nikon ACT-1 software (version 2.70).

4.4. Gene Expression Analysis

At the time of harvest, total RNA was extracted from jejunum and mid colon samples using the TripleXtractor reagent (Grisp, Porto, Portugal), in accordance with the instructions of the manufacturer. In total, 1 µg of the total RNA of each sample was reversely transcribed into cDNA using the GRS cDNA Synthesis Mastermix (Grisp, Porto, Portugal), in a total volume of 20 µL. cDNA templates were then amplified with specific primers for target

genes, *iNOS*, *NF-κB*, *NLRP3*, *TLR2* and *TLR4*, using Xpert Fast SYBR Mastermix (Grisp, Porto, Portugal) in a C1000 TouchTM thermocycler, equipped with a CFX96 Touch Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and analysed with the CFX MaestroTM software, version 2.3 (Bio-Rad Laboratories), in accordance with the protocol of the manufacturer.

Product amplification was performed with 1 µL of sample cDNA, corresponding to 50 ng of DNA, analysed in duplicate in a final volume of 20 µL/well, containing 10 µM of each primer (*iNOS* forward primer: 5'-CCAGAGCCTCATCGGTCGTC-3'; *iNOS* reverse primer: 5'-GGGTCCTTCCGCAGACAAC-3'; *NF-κB* forward primer: 5'-CCAGAGCC TCATCGGTCGTC-3'; *NF-κB* reverse primer: 5'-GGGTCCTTCCGCAGACAAC-3'; *NLRP3* forward primer: 5'-CCAGAGCCTCATCGGTCGTC-3'; *NLRP3* reverse primer: 5'-GGGTC CTTCCGCAGACAAC-3'; *TLR2* forward primer: 5'-CCAGAGCCTCATCGGTCGTC-3'; *TLR2* reverse primer: 5'-GGGTCCTTCCGCAGACAAC-3'; *TLR4* forward primer: 5'-CCAGAGCCTCATCGGTCGTC-3'; *TLR4* reverse primer: 5'-GGGTCCTTCCGCAGACAAC-3'). The PCR program run was as follows: (a) denaturation at 95 °C, 3 min; (b) amplification in 55 cycles (denaturation at 94 °C, 20 s; annealing at 55 °C, 30 s, and extension at 72 °C, for 30 s), followed by plate reads; (c) melt curve plotting between 65 °C and 95 °C, with 0.5 °C increments every 5 s, followed by plate reads. The change in gene expression was calculated using the $2^{-\Delta(\Delta C_t)}$ comparative method with the housekeeping gene 18S ribosomal RNA (18S rRNA) as the internal gene (18S rRNA forward primer: 5'-TTCCGGAAGTGAAGGCCATGATT-3'; 18S rRNA reverse primer: 5'-TTTCGCTCTGGT CCGTCTTG-3'), though the presented data were calculated by using the 18S rRNA gene normalized to the control group.

4.5. Correlation Analysis with Physiological Traits

Body VAT weight, adiposity index, and serum parameters in the end of dietary intervention were correlated (Pearson's correlation) with average daily intake of macronutrients.

4.6. Statistical Analysis

Data were tested for normal distribution and homogeneity of variances (Shapiro–Wilk test) and adjusted Welch test, when appropriate. The effects of treatment (HSD and WD) on all other variables were assessed by one-way analysis of variance (ANOVA). Pairwise comparisons between diet groups were determined either by the post hoc Tukey's test or the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. For correlation analysis, the Pearson correlation coefficients were used, with a confidence interval of 95%. The statistical analysis was performed using JASP open-source software (JASP, version 0.16.2.0, University of Amsterdam, Amsterdam, The Netherlands). A $p < 0.05$ was considered as statistically significant.

5. Conclusions

This study demonstrated that WD upregulates the *TLR2/TLR4/NF-κB* pathway axis and modulates intestinal *iNOS* expression, enhancing inflammatory response in the colon. The consumption of simple sugar activated a jejunal inflammatory response through the *TLR2/NF-κB* signalling pathway.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms231810984/s1>.

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1 Main results

The main results of the original research included in this Ph.D. thesis have shown that:

1.1 Obesity

Central obesity is more related to MetS than weight gain. There was an increase in adipose tissue content, namely in the abdominal adipose tissue weight, with both unhealthy diet groups revealing dysmetabolism.

1.2 Serum biochemical analysis

The results revealed that the serum levels of glucose, creatinine, AST and ALT, and lipid profile were increased in the rats of the HSD and WD groups. In addition, both dietary patterns were shown to be obesogenic, with a total of five dietary components correlating with blood lipid parameters. Total cholesterol levels are negatively correlated with complex carbohydrate intake. In addition, protein and fiber intake presented a negative correlation with triglyceride levels, whereas the mean fat energy, saturated fat, and sugar intake had a positive correlation.

1.3 Histomorphological changes in metabolic organs and intestine

Histopathological alterations were observed, including abnormal organization of hepatocytes and accumulation of lipid droplets in the liver, and abnormal structure of adipocytes. The intestinal morphology of the animals showed that HSD-fed rats exhibit enlargement of the villi and that enterocytes had misaligned nuclei and fewer intraepithelial lymphocytes compared to the control. In addition, vacuoles in absorptive enterocytes were observed in the small intestine, and the mucus-positive cell number per villi in rats fed with HSD was significantly higher.

1.4 Inflammation and oxidative stress in the liver

In both unhealthy diet groups, hepatic expression of TLR4, COX-2, and E-Selectin was increased, as well as oxidative stress biomarkers. The data obtained showed that unhealthy diets induce functional and structural changes in metabolic organs, suggesting pro-inflammatory and oxidative stress as potential mechanisms that trigger hepatic changes and metabolic dysfunction.

1.5 Inflammation in the intestine, location manner

The consumption of simple sugars caused a jejunal inflammatory response through the TLR2 / NF- κ B signaling pathway. In the jejunum, there was a significant variation in NF- κ B, TLR2, TLR4, and iNOS expression, but no difference in NLRP3 expression. Concerning WD, it induced the upregulation of TLR2, TLR4, and the NF- κ B pathway axis and modulated the intestinal iNOS expression, increasing the inflammatory response in the colon. Drinking a sweet beverage induced an inflammatory response in the small intestine through the activation of the TLR2-mediated NF- κ B signaling pathway, with consequent impaired intestinal barrier structure and function.

The results achieved can help develop new guidelines for eating habits and nutritional education, at younger ages and improve health and public health outcomes.

2 Discussion

Over recent years, increased attention has been devoted to children's and adolescent's nutrition to identify and prevent metabolic alterations that can extend to adulthood (De Marco et al., 2021; Lee & Yoon, 2018; Weihrauch-Blüher et al., 2019). Obesity is increasing in the early years of life and is considered an alarming condition. Eating healthy is highly conditioned by economic possibilities and by the cultural level of the entourage. Energy-dense foods are lower in price but less nutrient-rich. In addition, food consumption choices are affected by food availability and accessibility, which in turn are influenced by geography, demography, socioeconomic status, globalization, and marketing, for example (Serra-Majem et al., 2015).

Early-life consumption of high-fat and sugar-rich foods is recognized as a major contributor to the onset of metabolic dysfunction and its related disorders, including diabetes and non-alcoholic fatty liver disease (Cotter & Rinella, 2020a; Dowla et al., 2018). Nevertheless, the lifelong impact of early unhealthy eating habits remains unclear. To a better understanding of the influence of diet in triggering MetS, it is essential to understand the structural and functional changes in metabolic organs and the underlying pathological mechanisms. To investigate the long-term effects of early eating habits, young male rats were exposed to two unhealthy diets, which mimic the unhealthy dietary patterns in western society, namely a high consumption of sugary drinks and palatable and processed foods.

During the first years of life, high-caloric diets may not cause overweight (Pini et al., 2017). In accordance, young rats exposed to different unhealthy diets for 14 weeks did not have a marked increase in total body weight. Moreover, rats that had a high daily energy intake due to the consumption of sugar-rich beverages showed a significant decrease in final body weight. Nevertheless, there was a marked increase in visceral adiposity with both dietary patterns (Fig. 8). Because central obesity is associated with MetS, this exposes a convergence point in the MetS approach in childhood and adolescence. It also emphasizes that there's more than what meets the eye, because although one might think that there are no pathological effects from unhealthy dietary patterns because children and adolescents are not overweight, NAFLD and neuroendocrine disorders, might be already taking place, progressing silently.

One consequence of the exacerbated deposition of adipose tissue is dysregulation of the endocrine function (Xiao et al., 2020). It is known that leptin has metabolic effects in peripheral tissues, including stimulation of fatty acid oxidation (Angulo, 2002; Könner & Brüning, 2012b). In addition, it has been suggested that a high-fat diet may contribute to leptin resistance due to a defect in peripheral leptin's ability to activate hypothalamic areas (Ainslie et al., 2000; El-Haschimi et al., 2000). Plasma leptin concentrations can be influenced not only by total adiposity but also modulated by hormone starvation (Rayner & Trayhurn, 2001). Moreover, when there is insulin resistance, there is an increase in basal sympathetic activity (Russo et al., 2021). Leptin stimulates lipid utilization and lipolysis, possibly through the activation of sympathetic neurons that innervate adipocytes (Chrysafi et al., 2020). Increased levels of blood glucose and insulin cause damage to the vascular system, through sympathetic activation and increased oxygen requirements (Takei et al., 2022). Moreover, meal size influences the increase in plasma leptin (Perry et al., 2020), as well as the amount of secreted leptin, is directly proportional to the adipose mass (Xiao et al., 2020). Unexpectedly, neither HS nor WD diet groups had elevated leptin circulating levels, despite the higher adiposity index in both unhealthy diets. Thus, it can be hypothesized that there wasn't time to develop leptin resistance and consequently no hyperleptinemia was observed. Nevertheless, additional studies are required to address the impact of both diets on the sympathetic nervous system and their implications for insulin resistance at younger ages. As expected, serum biochemical parameters of young rats undergoing unhealthy diets were pathologically altered (Fig. 8). Regarding creatinine levels, it revealed an altered renal function (Schwartz et al., 1987). Concomitantly, there were alterations in lipid profile with significantly increased TAG, AST, ALT, total cholesterol, and glucose levels (Fig. 8). While one diet group had *ad libitum* access to a sweetened beverage, the other group, besides a sweetened beverage, also had access to palatable and processed foods with a high content of saturated fatty acids and sugar. The excessive consumption of sugary beverages by young children and adolescents may contribute to insulin resistance, as these added sugars have a more expressive glycaemic effect, and possibly activate endoplasmic reticulum stress with the ability to trigger a lipogenic program in the liver that promotes insulin resistance (Gluchowski et al., 2017; Gregor & Hotamisligil, 2011). Glucose availability is essential to lactate production (Arriarán et al., 2015; Sabater et al., 2014), but despite glucose increased levels, lactate production did not reflect it, in line with previous studies (Arriarán et al., 2015). It may

be explained by ADP availability as it appears to be the limiting factor for lactate production when glucose is at medium-high levels (Sabater et al., 2014). As a way of protecting the tissue from excess glucose and adipocyte hypertrophy resulting from excess energy substrates, redirection and a breakdown of glucose (6C) into two molecules of pyruvate (3C) can occur (Slawik & Vidal-Puig, 2007). This mechanism helps to protect against excess glucose but also causes hypoxia, which in turn can favor inflammation (Perry et al., 2020) and immune activation in adipose tissue (Sabater et al., 2014). Furthermore, the high ALT and AST circulating levels found are indicative of hepatocellular pathological alterations as they are widely used as biomarkers of liver injury (Angulo, 2002; Yang et al., 2014). The consumption of sweetened beverages and a high-energy diet can decrease urea cycle activity (Moreno-Fernández et al., 2018), which can justify the normal urea serum levels observed in unhealthy diets. Finally, lactate dehydrogenase (LDH) is a marker for diverse tissue injuries (Angulo, 2002), and is expected to be increased in MetS, but it wasn't elevated in any of the studied dietary patterns.

Regarding histochemistry analysis of liver samples, it is clear that NAFLD indicators were present. NAFLD is a hepatic manifestation with an increase in the intrahepatic lipid content, inflammation, fibrosis, and cirrhosis (Cotter & Rinella, 2020b; Peng et al., 2021b). Hepatic fibrosis was also observed in both unhealthy diets, being exacerbated in the group that had access to both sweetened beverages and high-energy foods. It was also evident an accumulation of lipid droplets and sinusoidal dilatation suggested that both diets may impair lipid metabolism, as it is known that a pathological lipid accumulation in the liver can lead to steatosis (Gluchowski et al., 2017). In addition, the expression of TLR4, E-Selectin, COX-2, and other inflammatory markers were elevated in both unhealthy diet groups, as well as the oxidative stress biomarkers (Fig. 8). Expression of TLR4 can respond by local or systemic inflammation, through activation of NF- κ B and production of pro-inflammatory cytokines. As a consequence, there is a decrease in insulin sensitivity and an increase in COX-2 expression (Francés et al., 2015; Motiño et al., 2019). Despite an increase in hepatocyte COX-2 protein expression, no significant increase in liver weight between animals was observed. COX-2 expression might have a counterbalancing effect in the prevention of lipid accumulation in the liver and the worsening effect on serum lipid profile induced by early unhealthy eating habits (Martín-Sanz et al., 2010).

An increase of hepatic pro-inflammatory molecules and E-Selectin, induce the activation of TLR4, causing OS and an increase in profibrogenic signals, leading to liver fibrosis (Ignat et al., 2020). The present results support previous findings by showing that young rats undergoing HSD and WD diets show an increase in TLR4 expression, increased ALT levels, and liver E-Selectin expression, accompanied by an increase in hepatic lipid peroxidation (Rodrigues et al., 2021; Simons et al., 2020). E-selectin is a vascular cell adhesion molecule, up-regulated in inflammation, that has been looked at as having a key role in steatosis and NASH (Rodrigues et al., 2021). Hepatic E-Selectin expression was present and increased in the vascular endothelium of HSD and WD. It is an inflammatory marker specific to the blood vessels (Simons et al., 2020). It is expressed on cytokine-activated endothelial cells of hepatocytes, as a consequence of systemic inflammation or liver injury (Rodrigues et al., 2021). Inflammation is also linked to OS, and both seem to share TLR signaling pathways. Moreover, OS activates inflammatory kinases that cause vascular dysfunction as a response to metabolic alterations, possibly leading to metabolic diseases such as T1D and NAFLD (Porrás et al., 2017b; Silva et al., 2018). Lipid peroxidation, as a marker of OS, was increased in both unhealthy studied diets, but significantly higher in the WD group, likewise the tendency in inflammatory markers expression. Other OS-pronounced actions have been shown on adipose tissue as they induced the differentiation and proliferation of adipocytes, stimulated their deposition, and triggered an inflammatory response that disrupts insulin signaling (Johnson et al., 2016; Kelly et al., 2016).

In obesity, macrophages increase in number and, unlike in normal-weight individuals, they change to M1 pro-inflammatory status (Boulenouar et al., 2017). The shift to an inflammatory phenotype promotes the development of persistent local inflammation in which various biologically active molecules are released (Zatterale et al., 2020). The relationship between adipose tissue inflammation and other pathological features of obesity is not well understood. However, increased adipose tissue inflammation appears concomitantly with increased systemic inflammation, reduced insulin sensitivity, and decreased muscle oxidative capacity. Together, it suggests that adipose tissue inflammation links to systemic inflammation and other systemic and peripheral metabolic disorders associated with increased adiposity, such as reduced insulin sensitivity and mitochondrial respiration of skeletal muscle (Kunz et al., 2021). The results obtained showed that animals subjected to the WD had abnormal

adipocyte structure, namely an increase in the mean area, which is indicative of adipocyte hypertrophy. Hypertrophic adipocytes have a higher rate of death and altered secretion of chemoattractant proteins (Heilbronn & Liu, 2014; Jernås et al., 2006). In turn, there are no significant differences in the mean adipocyte area of animals on the sugary diet. Nevertheless, there are abnormal changes in the shape of the adipocytes, which may suggest that some pathological changes may occur within the extracellular matrix. The extracellular matrix of adipose tissue is essential for the adipocyte's function and remodeling to achieve the necessary adaptation to energy stores (Rutkowski et al., 2015). It is sensitive to lipid accumulation resulting from obesity. When there is a positive energy balance, there is an accumulation of extracellular matrix, contributing to fibrosis (Lee et al., 2014), which can cause necrosis and attraction of pro-inflammatory macrophages (Lin et al., 2016). The adipose tissue undergoes remodeling that is pathologically accelerated in the obese state promoting the activation of inflammatory signaling pathways, including NF- κ B, endothelial adhesion molecules, and chemotactic mediators (Blüher et al., 2009). Low-grade systemic inflammation is seen as a possible link between obesity and insulin resistance (Weisberg et al., 2003; Xu et al., 2003). Other studies have associated hypoxia, which occurs in the adipose tissue during obesity, with insulin resistance (Regazzetti et al., 2009; Yin et al., 2009). Nevertheless, the size of visceral adipocytes is negatively correlated with insulin sensitivity, leading to the consideration that there is a correlation with insulin resistance (Hardy et al., 2011; O'Connell et al., 2010). The adipose tissue is one of the three major insulin-sensitive tissues, alongside the skeletal muscle and the liver. In an insulin resistance scenario, pancreatic β -cells compensate by increasing insulin secretion to restore blood glucose levels. A continuous decline in insulin sensitivity extenuates these β -cells, resulting in hyperglycemia and possibly T1DM (Shulman, 2000). Although the observed changes in adipose tissue architecture suggest alterations in the extracellular matrix, their contribution to the aforementioned effects on chronic low-grade inflammation that is associated with insulin resistance requires further elucidation.

Histopathological examination of intestine samples exhibited similar pathological changes but more severe in WD (Fig. 8). The morphological changes observed in the intestines of HSD and WD animals have reported pathological alterations, namely in duodenal villi characteristics. The animals submitted to the standard diet showed slender duodenal villi with

a regularly aligned epithelium showing columnar cells with enterocytes interspersed by goblet cells and disperse intraepithelial lymphocytes. In the HSD group, there was an enlargement of the villi, the enterocytes revealed misaligned nuclei, and vacuoles in absorptive enterocytes were present in the duodenum. In rats fed with the HSD diet, intraepithelial lymphocytes were present in smaller numbers considering the tip-to-villi base, whereas the mucus-secreting cells per villi were significantly higher. Previous studies have also shown that mice on an HSD exhibited glucose homeostasis impairment and obesity, disruption of intestine epithelium integrity, and systemic inflammatory response. There were significant differences in the number of goblet cells, known as mucus-producing cells, between rats fed with HSD and WD, but not compared with the control feeding group. Goblet cells are under the regulation of the immune system and respond to different stimuli depending on the region (Birchenough et al., 2015). The NLRP3 inflammasome has caught the attention of investigators, as an immune cell sensor involved in inflammatory pathways with possible involvement in metabolic dysfunction (meta-inflammation). Particularly in the case of a high-fat diet, NLRP3 seems to have a favorable effect on insulin resistance and obesity-related adipose tissue inflammation (Ralston et al., 2017; Vandanmagsar et al., 2011), through upregulation of TLR4 and TLR2 signaling (Caesar et al., 2015). NLRP3 may potentially have a regulatory role in metabolic inflammation because it can be induced by microbial components, TLR ligands, and transcription factor NF- κ B, but also by fatty acids (Franchi et al., 2012; T. Liu et al., 2017a; Ralston et al., 2017; Zhen & Zhang, 2019). Together, it suggests that NLRP3 can have a role in immune dysfunction and diet-induced obesity and insulin-resistance (Franchi et al., 2012; Ralston et al., 2017; Vandanmagsar et al., 2011; Zhen & Zhang, 2019). Nor WD nor HSD feeding influenced the expression of the NLRP3 gene either in the small or in the large intestines, although it can't be excluded the possibility of inflammasome activation by other stimuli. One possible explanation is that the intestine, unlike other organs, has tissue-resident phagocytes that are hyporesponsive to microbial stimulation (Franchi et al., 2012), which should be seen as a protection for the host, having a tolerance threshold to non-pathogenic microbiota.

The TLR2-mediated NF- κ B signaling pathway was triggered in the small intestine by sweet beverage consumption, contributing to an impaired barrier structure and function. TLR2 and TLR4 are expressed in immune and non-immune cells throughout the intestinal epithelium, in

a specific region distribution, and in enteric neurons (Anitha et al., 2016; Burgueño & Abreu, 2020; Hörmann et al., 2014; Paone & Cani, 2020; Paveljšek et al., 2021; Price et al., 2018; Seguella et al., 2021). Expression of TLR4 appears to increase in the gut with the consumption of a high saturated fatty acids diet, in a way that depends on the gut location (Kim et al., 2012); regarding TLR2 there is less information about the influence of an obesogenic diet in its expression. However, some authors propose that the fatty acid-specific effect on intestinal inflammation occurs with the activation of TLR2 and TLR4 signaling pathways, causing low-grade inflammation and glucose homeostasis impairment (Rohr et al., 2018; Velloso et al., 2015). The role of TLR2 is crucial in the regulation of the intestinal barrier (Burgueño & Abreu, 2020). Its expression is more pronounced in the proximal colon, being influenced by GM (Price et al., 2018). However, feeding with HSD induced the activation of TLR2/NF- κ B only in the small intestine. The overexpression of TLR2 changes tight junction protein levels with ensuing alterations in tissue integrity and mucus secretion by goblet cells (Burgueño & Abreu, 2020), as verified by the discovery of morphological changes in the small intestine. A persistent high luminal glucose concentration can lead to the upregulation of TLR2 expression in the small intestine, altering intestinal permeability and causing hyperglycemia (Thaiss et al., 2018). The results achieved showed that WD caused the activation of the TLR2/TLR4/NF- κ B signaling cascade, inducing an inflammatory response in the small intestine, but a more exacerbated expression in the colon. In addition, a high-fat intake leads to the upregulation of iNOS only in the colon, which in turn, leads to the increase of NO production, an immunomodulator of gastrointestinal motility.

The gut-brain axis is influenced by GM metabolites like short-chain fatty acids. Butyrate is an important short-chain fatty acid as it inhibits histone deacetylases and thus stimulates memory and synaptic plasticity (Vecsey et al., 2007), alongside influencing enteric serotonin. Fibre intake influences microbiota-host interactions, interfering with overall metabolic function and, locally, modulating intestinal homeostasis (An et al., 2021). Since both obesogenic diets studied had a low-fiber content, the deregulation of the iNOS signaling pathway caused by saturated fat might be an internal signal to disrupt microbiota-host interactions, contributing to the colonic inflammatory response and dysmotility reported. Animal models of colitis suggested the link between the expansion of *Proteobacteria* and colonic inflammation. In a previous study with this animal model, it was shown that the

consumption of ultra-processed foods induced the expansion of *Proteobacteria*, supporting the hypothesis that the iNOS expression in the colon can be a consequence of a disruption of microbiota-immune-nerve crosstalk (Bódi et al., 2019; Grubišić & Gulbransen, 2017; Litvak et al., 2017; MacEachern et al., 2015). Notwithstanding, the downregulation of jejunal iNOS expression observed in rats fed with WD and HSD can be due to differences in animal strains and the nutrient composition, or the segment of the intestine used in each animal. The decrease of iNOS expression in the small intestine with the two unhealthy diets may be explained by a possible chronic defect in the immune response, caused, in its turn, by diet effects on the jejunal bacterial load.

Diet can also influence the overall proliferation of enterochromaffin cells, through the activation of TLR2. Therefore, it is expected that the total amount of serotonin might also be increased. Enteric serotonin is a neuromodulator of intestinal physiology and a signaling molecule that takes part in several physiological functions (Mawe & Hoffman, 2013). The consumption of unhealthy diets causes an increase in the number of enterochromaffin cells in the small intestine, particularly a diet with a high-fat content (Linan-Rico et al., 2016; Lumsden et al., 2019; Reigstad et al., 2015a). It increases tryptophan expression, serotonin tissue levels, and free serotonin levels in portal blood. Altogether, it contributes to the development of NAFLD. The diversity and stability of the GM can be affected by high-fat diets or high simple sugar diets leading to dysbiosis, which is an alteration observed in obesity. A dysbiotic microbiota is thought to alter the communication between the gut and the brain axis contributing to mood (e.g., anxiety, depression, sensitivity to stress) and cognitive alterations, consequently leading to impaired memory and reduction of attention. In infants and adolescents, there are pronounced central nervous system changes in key brain structures, such as the amygdala and hippocampus and the function of these regions is dependent on serotonergic neurotransmission (Choi et al., 2018). Tryptophan availability and peripheral serotonin seem to influence energy metabolism, causing, for example, lipid accumulation in hepatocytes (Choi et al., 2018). To this point, it is now known that HSD and WD diets cause an augment of intestinal inflammatory response and unfavorable metabolic profile (adiposity and biochemical parameters), accompanied by abnormal accumulation of lipids in the hepatocytes. In addition, dysbiosis has been linked to several systemic alterations, including its influence on the function of serotonergic systems, which is pointed to have a key

role in the onset of depression, underlining the intimate linking that connects both the gut and the brain (Reigstad et al., 2015). It has been shown that a fat-rich diet increases tryptophan expression and tissue serotonin levels in the gut, and free serotonin levels in portal blood (Bertrand et al., 2011). Moreover, the deregulated production of enteric serotonin has been linked to several diseases (Sikander et al., 2009). It underlines the importance of future studies to quantify the tissue serotonin and tryptophan levels and establish a complete profile of the kynurenine pathway in the periphery and the central nervous system. Future studies should also address how high-energy diets affect gut permeability, specifically, gut tight junction protein expression in infancy and adolescence. Moreover, the direct role of enteric serotonin in the regulation of the hepatic lipid metabolism *in vivo*, and elucidation on the exact contribution of central and peripheral serotonin systems in cognitive dysfunction and anxiety-like behaviors should also be addressed.

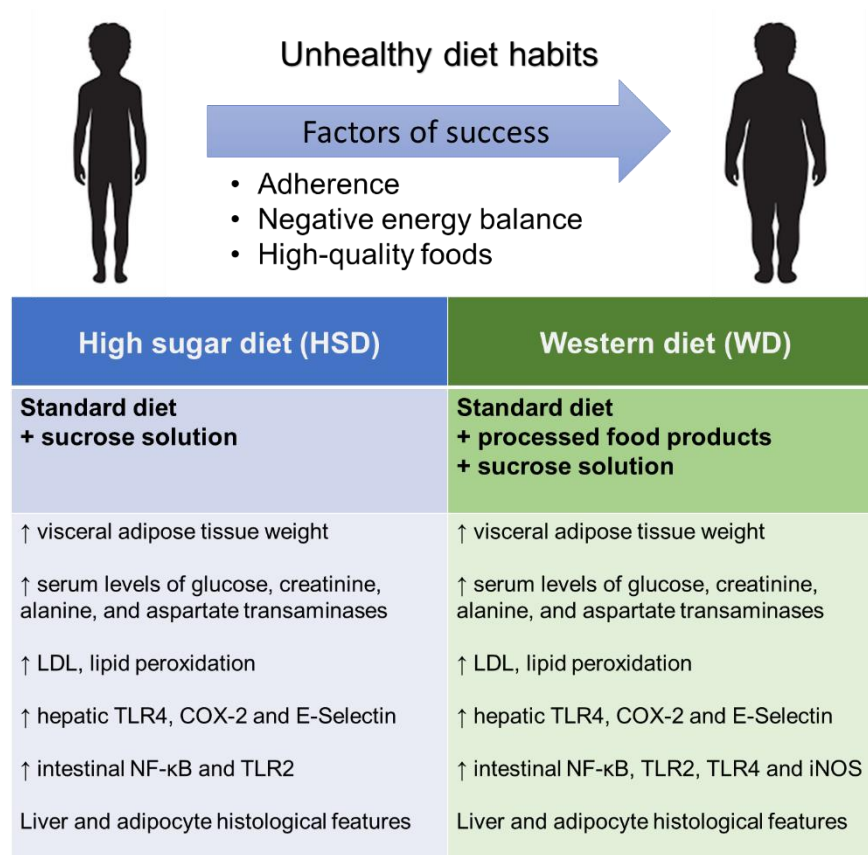


Figure 8 – Immunometabolic, inflammatory and histomorphometric profiles of HS and WD diets. There was an increase in adipose tissue content and serum pathological alterations in lipid profile as well. Within the liver, the expression of TLR4, COX-2 and E-Selectin was also altered in both HS and WD diets compared to control. In turn, in intestine, HS induced alterations in the expression of NF-κB and TLR2, whereas WD also induced alterations in iNOS and TLR4. In addition, the histopathological changes were further observed in the liver and adipose tissue. HS: high-sugar diet; WD: western diet; LDL-C: low-density lipoprotein cholesterol; TLR4: Toll-like receptor 4; COX-2: cyclooxygenase 2; NFκB: nuclear factor kappa B; TLR2: Toll-like receptor 2; iNOS: inducible nitric oxide synthase.

3 Conclusions

A precocious identification of MetS risk in pediatrics is of extreme importance. Many definitions of MetS in children and adolescents have been suggested, but there is no unified definition to assess the risk or the outcomes. Disorders of glucose and lipid metabolisms can affect a myriad of organs and tissues, under insulin-resistant conditions, outbursting in the observed interplay of the various metabolic abnormalities.

HSD and WD, at a young age, induced functional and structural changes in the liver and adipose tissue, increased inflammation, and OS, central obesity, triggering the mechanisms leading to other manifestations of MetS (Fig. 8). There was a marked increase in the adiposity index with the consumption of HSD and WD. The WD induced a worse metabolic profile, accompanied by changes in the adipocytes and hepatocyte normal structure, with an increase of inflammation and OS in the liver. The colonic inflammatory response due to the consumption of ultra-processed foods might play a key role in MetS pathogenesis identified in adulthood. The effects induced by sugar beverages on inflammation pathways occur mainly in the small intestine. The intestinal structure and its regulatory signaling pathways can be disrupted by direct and indirect effects of different nutrients and in a regional-specific manner, providing support for the interplay between early consumption of unhealthy diets and low-grade chronic inflammation, as a crucial trigger of obesity in later life.

Diet affects the intestinal barrier integrity, under influence of GM and this encapsulates the pathophysiology of several other extra-intestinal diseases, such as NALD, TIID, CVD, as well as brain diseases. The dynamic changes in GM related to dietary patterns and microbiota-derived metabolites can alter the communication in the gut-brain axis, contributing to abnormal brain function and behavior. Thus, it is important to clarify the connections between unhealthy diets and metabolic syndrome, and cognitive impairment. In the future, circulating inflammatory biomarkers may be used for risk assessment of metabolic diseases, justified by the strong association between chronic inflammation and obesity. Early detection and treatment, particularly with a lifestyle intervention, are crucial to stop the progression of MetS in children and adolescents.

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