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Association between individual phenotypic features and lifestyle factors on the risk of non-alcoholic fatty liver disease in older subjects with metabolic syndrome

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"Yo planté, Apolos regó; pero el crecimiento lo ha dado Dios".

Corontios 3:6

A mis padres y hermanos

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"There is a driving force more powerful than steam, electricity and atomic energy: will."

Albert Einstein

LIST OF ABBREVIATIONS

AHA	American Heart Association
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
apoB	Apolipoprotein B
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
ATP III	National Cholesterol Education Program
AUC	Area under the curve
BCRP	Breast cancer resistance protein
BMI	Body mass index
CHD	Coronary heart disease
ChREBP	Carbohydrate response element binding protein
CPT	Carnitine palmitoyltransferase
CT	Computed tomography
CYP2E1	Cytochrome P450 Family 2 Subfamily E Member 1
CVD	Cardiovascular diseases
DASH	Dietary Approaches to Stop Hypertension
DHR	Dihydroresveratrol
DNL	De *novo* lipogenesis
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	Endoplasmic reticulum
EVOO	Extra virgin olive oil
FADH2	Flavin adenine dinucleotide
FFA	Free fatty acid
FFQ	Food frequency questionnaire

FGF-21	Fibroblast growth factor 21
FGIR	Fasting glucose-insulin ratio
FIB-4	Fibrosis-4 score
FIRI	Fasting insulin resistance index
FLI	Fatty liver index
GGT	Gamma-glutamyl transferase
GLUT	Glucose transporter
^{1}H-MRS	Proton magnetic resonance spectroscopy
HbA1c	Haemoglobin A1c level
HCC	Hepatocellular carcinoma
HDL-c	High-density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HOMA-%B	Homeostasis model assessment for β-cell function
HOMA-IR	Homeostasis model assessment of insulin resistance
HSC	Hepatic stellate cells
HSI	Hepatic steatosis index
IDF	International Diabetes Federation
IL	Interleukin
JNK	c-Jun N-terminal kinase
LC–MS	Liquid chromatography–mass spectrometry
LDL-c	Low-density lipoprotein cholesterol
LFD	Low fat diet
LPL	Lipoprotein lipase
MEDAS	Mediterranean Diet Adherence Score
MedDiet	Mediterranean diet
MET	Metabolic equivalent task
MetS	Metabolic syndrome
MRI	Magnetic resonance imaging
MRP	Multidrug resistance protein

MS	Mass spectrometry
MTP	Microsomal triglyceride transfer protein
MUFAs	Monounsaturated fatty acids
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFS	NAFLD fibrosis score
NHLBI	National Heart, Lung, and Blood Institute
NO	Nitric oxide
oxLDL	Oxidized low-density lipoproteins
PPAR-α	Peroxisome proliferator activated receptor alpha
PREDIMED	Prevención con dieta Mediterránea
PUFAs	Polyunsaturated fatty acids
QqQ	Triple quadrupole
REGICOR	Registre Gironi del Cor questionnaire
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRR	Relative risk ratio
SAT	Subcutaneous fat
SREBP	Sterol regulatory element-binding protein
SULT	Sulfotransferase
T2DM	Type 2 diabetes mellitus
TNF-α	Protein tumor-necrosis factor-α
TyG	Triglyceride and glucose index
UGT	UDP-glucuronosyltransferase
UPLC	Ultra-performance liquid chromatography
UPR	Uncoupled protein response
VAT	Visceral adipose tissue
VLDL-c	Very-low-density lipoprotein cholesterol

LIST OF PUBLICATIONS

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease, whose prevalence is growing worldwide in parallel with obesity and type 2 diabetes mellitus (T2DM) rates. This trend spreads the global burden of NAFLD due to an increase in health-care costs and a negative impact on wellbeing and the quality of life. Moreover, NAFLD are strongly associated with components of the metabolic syndrome (MetS), including central adiposity, atherogenic risk profile, insulin resistance, and hypertension.

The prevalence of NAFLD is higher in men than women in adulthood. However, this tendency changes with ageing, because the postmenopausal status is associated with a higher risk to develop NAFLD. Besides that, an enlarged and disrupted visceral adipose tissue (VAT) is also present, leading to insulin resistance, which plays a central role in the pathogenesis of NAFLD.

Unhealthy lifestyle behaviours, including poor dietary quality and low physical activity, cause harmful effects on health outcomes. The associations between sex-differences, metabolic phenotype, lifestyle factors and specific bioactive compounds on risk factors and predisposition to develop NAFLD in the elderly population with high risk of cardiovascular disease and MetS features are scarcely known.

Hence, the aims of the present thesis were: 1) to evaluate sex-related differences in risk factors of NAFLD in elderly individuals; 2) to assess the relationship between VAT and insulin resistance on NAFLD risk factors; 3) to study the associations between lifestyle factors focused on adherence to the Mediterranean diet (MedDiet), dietary components, and physical activity on the risk to develop NAFLD; 4) to investigate the interrelationships between some urinary metabolites associated to MedDiet with cardiometabolic and liver markers involved in NAFLD development.

The main results achieved by this study in ***Chapter 1*** was that increased risk to develop NAFLD in women compared to men in older individuals diagnosed with MetS was found. In ***Chapter 2***, participants with higher VAT deposition presented a worst atherogenic profile, impaired glucose metabolism and more disturbed liver functions. This research also found that increased TyG values were positively associated with non-invasive liver markers. Moreover, the TyG index could be a simple surrogate marker of increased VAT in women with MetS features. In ***Chapter 3***, those individuals with greater adherence to the MedDiet and increased levels of physical activity presented lower hepatic steatosis index (HSI) related to oxidative and liver steatosis state. Furthermore, higher consumption of legumes was associated with a lower risk of increased HSI values. In ***Chapter 4***, a healthier lipid

profile and liver enzymes levels were linked to higher concentrations of urinary resveratrol metabolites, where total urine resveratrol concentration was associated with lower risk of elevated hepatic enzymes.

In summary, findings presented in this thesis suggest the potential influence of sex-related differences, insulin resistance, and dysfunctional VAT on the predisposition of NAFLD contributing with a little more scientific knowledge to better understand the pathological crosstalk between NAFLD and MetS. Notably, the TyG index could be proposed as a reliable marker of increased VAT content in women with MetS features. These results are essential, considering that the metabolic heterogeneity in NAFLD risk factors is crucial in the identification of high-risk individuals and the management of NAFLD. Another point of interest is that lifestyle modifications focused on adherence to the MedDiet, especially promoting the consumption on some dietary components and bioactive compounds related to resveratrol as well as physical activity and might be considered relevant in the prevention and treatment of NAFLD in elderly individuals at high cardiovascular risk diagnosed with MetS.

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I.   INTRODUCTION

INTRODUCTION

1. NON-ALCOHOLIC FATTY LIVER DISEASE

    1.1. Definition and clinical features

Non-alcoholic fatty liver disease (NAFLD) is defined as an excessive accumulation of intrahepatic lipid content (exceeding 5% of total liver weight) in patients without significant alcohol consumption (≥30 g for men and ≥20 g for women) and the exclusion of secondary causes of liver fat steatosis [1–4] (Table 1). NAFLD encompasses a spectrum of fatty liver diseases, which in early stages, initiate with non-alcoholic fatty liver (NAFL) characterized by simple steatosis that can progress into non-alcoholic steatohepatitis (NASH) [1]. Increased portal hypertension and hepatocyte damage trigger to the activation of hepatic stellate cells that stimulates collagen deposition in the hepatic tissues where its replaced by fibrotic scarring [1,3]. In the end-stage, NAFLD can eventually lead to cirrhosis and hepatocellular carcinoma (HCC) [1,3] (Table 2).

Table 1. Hereditary, nutritional, drug treatment and other conditions associated with secondary hepatic steatosis

Inborn errors of metabolism	Nutritional	Drugs	Others
Disorders related to lipid metabolism	Marasmus	Amiodarone	Inflammatory bowel disease
Abetalipoproteinemia	Kwashiorkor	Perhexiline maleate	
Hypobetalipoproteinemia	Starvation		HIV and hepatitis C infection
Familial combined hyperlipidaemia	Obesity	Methotrexate	
Glycogen storage disease	Diabetes Mellitus	Carbamazepine	Small intestinal bacterial overgrowth
Galactosemia	Dyslipidaemia	Valproic acid	
Lipodystrophy	Total parenteral nutrition	Tetracycline	Celiac disease
Tyrosinemia		Tamoxifen	Surgical weight loss procedures (bariatric or Jejunoileal surgery)
Hereditary fructose intolerance		Corticosteroids	
Systemic carnitine deficiency			
Homocystinuria			
Weber-Christian syndrome			
Wilson's disease			

Abbreviations: human immunodeficiency virus, HIV.

Table 2. Spectrum of Non-alcoholic Fatty Liver Disease

NAFLD progression	Definition
Non-alcoholic Fatty Liver (NAFL)	Presence of ≥5% fat droplets into hepatocyte without evidence of liver damage (ballooning or fibrosis).
Non-alcoholic steatohepatitis (NASH)	Liver steatosis accompanied by hepatic lesions (intralobular inflammation, ballooning with or without fibrosis). This stage can progress to cirrhosis and liver failure.
NASH cirrhosis	Presence of cirrhosis (scarring of the liver) with current or previous histological signal of steatosis or steatohepatitis.
Cryptogenic Cirrhosis	Cirrhosis of uknown etiology. Indivuduals presented comorbidities associated with NAFLD.

1.2. Prevalence, trends and economic burden

NAFLD is the most common chronic liver disease worldwide [1]. Their incidence increases in parallel with the prevalence of obesity, type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVD), and metabolic syndrome (MetS) features [1,2,5]. NAFLD rates are estimated at 24% around the world, evidencing a higher prevalence in South America (31%) and the Middle East (32%), Asia (27%), USA (24%), and Europe (23%) with less incidence in Africa (14%) [5]. The reported prevalence of NAFLD varies widely depending on the ethnic populations, geographic regions, and the diagnosed used [5,6]. Higher prevalence of NAFLD is widespread in individuals with obesity, T2DM and dyslipidaemias [1,2,6]. In a study, Younossi et al. 2016 showed that 51.3% of the population diagnosed with NAFLD and 81.8% from NASH had obesity [6]. Also, the incidence of T2DM was presented in 22.5% and 43.6% in individuals with NAFLD and NASH, respectively [6]. Furthermore, the presence of hyperlipidaemia/dyslipidaemia was diagnosed in 69.2% and 72.1% among NAFLD and NASH patients [6]. It has suggested that around 10 to 29% of patients diagnosed with NASH may have liver cirrhosis within ten years [3]. Meanwhile, between 4-27% of these individuals may have hepatocellular carcinoma [3]. Data reported that liver mortality among individuals with NAFLD was 0.77 per 1,000 person-years and 11.77 per 1,000 person-years for patients with NASH [6]. These raising trends have a negative impact on economic and clinical burden [5,7].

It is estimated that U.S population diagnosed with T2DM and NAFLD/NASH, the cost for NAFLD in 20 years were $55.8 billion 7. Likewise, individuals with NASH and T2DM will account for 65,000 transplants, 1.37 million CVD deaths, and 812,000 liver-related deaths [7]

1.3.   Pathogenesis: the hits hypothesis

An established theory proposed that the development of simple steatosis to NASH follow two-hit model [8]. The first hit is characterized by an excessive accumulation of lipids in hepatocytes increasing the risk of developing the second hit that is a state of oxidative stress and mitochondrial dysfunction, which leads to an increased reactive oxygen species (ROS) production. Thus, the second hit has been a requisite to progress from NAFLD to NASH, and eventually to cirrhosis [8].

In this context, several mechanisms are involved in NAFLD pathogenesis (Figure 1), including insulin resistance, a lipodystrophic state that promote hepatocyte susceptibility to oxidative stress, and the activation of several pathways related to inflammation and apoptosis, which we will review into hits hypothesis.

1.3.1.   First hit: Lipid accumulation in hepatocytes

The excessive hepatic fat accumulation is derived from several sources characterized by an excessive lipid deposition in the liver. Donnelly et al. quantified the principal sources of hepatic and triacylglycerol in patients with NAFLD by the method of multiple stable isotopes [9]. Authors showed that around 60% of triglyceride is derived from non-esterified fatty acids, which are products of lipolysis from adipose tissue, 26% from de *novo* lipogenesis (DNL), and 15% from the diet. These findings indicate that a disruption in peripheral fatty acid flux and DNL increases hepatic fat accumulation in NAFLD [9].

Steatosis occurs when there is an imbalance between the rate of lipid input and the rate of lipid output. In addition, there are many pathways which allow an increased influx of triglycerides in the hepatocytes [10], including the hepatic fatty acid uptake (increased synthesis), *de novo* fatty acid synthesis (increased delivered), the fatty acid oxidation (reduced fatty oxidation), and the fatty acid export (reduced very-low-density lipoprotein cholesterol (VLDL) secretion).

1.3.1.1. Fatty acid uptake

Insulin stimulates the activity of the adipocyte lipoprotein lipase (LPL), an enzyme which hydrolyzes triglycerides in lipoproteins to free fatty acid (FFA) [11]. Thus, FFAs are mobilized

from the white adipocytes under fasting conditions via lipolysis by the action of the hormone sensitive lipase in order to export FFA into the plasma [11]. In a condition of insulin resistance, these abilities are reduced due to the increased rate of lipolysis and raised FFA plasma levels increasing the risk to develop hepatic steatosis [11]. Nielsen et al. evaluated the contribution of visceral adipose tissue (VAT) lipolysis to hepatic FFA delivered to lean and obese subjects by isotope dilution hepatic vein catheterization techniques [12]. This study revealed that levels of FFA in plasma are 20% greater in obese subjects compared with lean subjects. In subjects with less visceral fat, around 5% of the hepatic FFA is derived from adipose tissue lipolysis [12]. Supporting this finding, Fabbrini et al. reported FFA kinetics in obese subjects with NAFLD with normal (≤5.5%) or high (>10%) amount of intrahepatic triglyceride content determinate by magnetic resonance spectroscopy. The hyperinsulinemic clamp procedure and the deuterated palmitate tracer infusion were performed to measure the FFA kinetics. The results showed high adipose tissue lipolysis among obese subjects with a high amount of intrahepatic triglyceride content during fasting and feeding conditions. This observation indicates an increase of the FFA flux into plasma during the day [13]. Genes related to lipid metabolism and fatty acid transport are upregulated in subjects with NAFLD. In this context, a study was conducted to investigate the gene expression on liver samples from individuals diagnosed with NAFLD and with a severe degree of steatosis without histological signs of inflammation compared to individuals with low lipid fat accumulation. The expression of Fatty-acid-binding protein 4 adipocyte and CD36 molecule (thrombospondin receptor), which are genes related to lipid metabolism, were correlated positively with liver fat content. Taken together this shows a reduced efficacy of lipolysis and an increased fatty acid uptake might be associated with hepatocellular lipid accumulation, and can lead to a progression of liver disease [14].

1.3.1.2. De novo fatty acid synthesis

De novo lipogenesis (DNL) is a process that mainly occurs in the liver or adipose tissue. These metabolic steps are taking place in the cytoplasm (mitochondria), where the excess of carbohydrate is converted into fatty acids and esterified into triglyceride. DNL starts with the conversion of acetyl-CoA into malonyl- CoA by acetyl-CoA carboxylase, which involves several cycles and reactions [15]. The malonyl-CoA is used as a substrate in order to produce 16-carbon palmitic acyl-CoA by the enzyme fatty acid synthase, which plays a pivotal role in fatty acid synthesis [15]. Thus, the contribution of fatty acids via the DNL pathway in NAFLD is important. Indeed, a study has shown that patients with higher fatty liver content have more than threefold rates of fatty acid synthesis via DNL compared with individuals with

lower fatty liver amount [16]. Furthermore, postprandial blood glucose and insulin levels are rising and promoting the hepatic lipogenesis. This state activates the principal pathways involved in the regulation of DNL, which are the sterol regulatory element-binding protein (SREBP) that is stimulated by the effect of glucose, and the carbohydrate response element binding protein (ChREBP) activated by insulin. These transcription factors increase the expression of glycolytic and lipogenic genes [17]. SREBPs are responsible for activating almost 30 genes related to lipid metabolism on hepatocyte cells involved in DNL. Three isoforms of SREBPs, the SREBP-1a, SREBP-1c, and SREBP-2 are encoded by the human genome. Thus, the SREBP-1c is responsible for modulating genes related to fatty acid and triglyceride metabolism, whether the SREBP-2 modulates genes involved in cholesterol metabolism [18].

A review has been published summarizing the evidence from several studies in rodents to determine the action of the transcription factor SREBP-1c in DNL and hepatic steatosis. In the livers of ob/ob or lipodystrophic mice with severe hepatic insulin resistance, hyperglycaemia, and steatosis, the hepatic levels of SREBP-1c are disrupted. Authors have also suggested that insulin resistance and especially the ER stress induces hyperstimulation in the lipogenic pathway by inducing SREBP-1c, explaining the "paradoxical stimulation of lipogenesis in an insulin-resistant liver" [19]. Thus, activation of SREBP-1c increases malonyl-CoA in the mitochondrial membrane by inhibiting β-oxidation of FFAs, and is directed towards the formation of triglycerides. This metabolic situation increases the production and accumulation of lipids in a hepatocellular level, leading to hepatic steatosis [20]. For ChREBP, evidence suggests that this molecule/gene plays a crucial role in the development of steatosis. Dentin et al. showed a higher liver gene expression of ChREBP in leptin deficient ob/ob mice, and silencing ChREBP expression diminished liver steatosis significantly [21]. Linked to this, VAT promotes the alteration of glucose and lipid metabolism by increasing the production of FFAs in the liver (DNL) by overexpression of these transcription factors [20]. Keeping with the contention that SREBP-1c and ChREBP are involved in the pathogenesis of NAFLD, high levels of postprandial glucose and insulin postprandial develop hepatic fat accumulation.

1.3.1.3. Fatty acid oxidation

Fatty acid oxidation is a major source of adenosine triphosphate (ATP) in tissues such as liver, skeletal muscle, and heart; especially in fasting conditions where glucose availability is limited, fatty acids are used as main source [22]. Thus, fatty oxidation can occur in the mitochondria, peroxisomes, and endoplasmic reticulum (ER). Under an excessive flux of FFA and elevated insulin resistance, oxidation of long-chain fatty acids in peroxisomes and

ER leads to an overproduction of ROS and lipotoxicity [23]. The principal pathway is β-oxidation, which is a cyclic process where fatty acids of short, medium, and long chain are broken down within mitochondria. Acyl-CoA (long chain) is converted to acylcarnitine by the action of carnitine palmitoyltransferase (CPT) 1, which occurs in the outer mitochondrial membrane, and then is shuttled inside by carnitine acylcarnitine translocase. Finally, CPT 2 reconverts the acylcarnitine into acyl-CoA, which is able to enter inside the mitochondria matrix in order to initiate the β-oxidation. This process is necessary for long chain fatty acids such as palmitoyl-CoA, oleoyl-CoA, and linoleoyl-CoA, but short- and medium-chain length are able to penetrate the mitochondrial membrane by diffusion and may not need the action of carnitine [22]. This process includes several complex cycles of dehydrogenation, hydration, and cleavage reactions that depend on the length of the fatty acid [22]. Each cycle shortens the fatty acyl-CoA by two carbon atoms, acetyl-CoA, one flavin adenine dinucleotide (FADH2), and one nicotinamide adenine dinucleotide (NADH) that are released as acetyl-CoA [22]. The acetyl- CoA generated enters in the Krebs cycle for oxidation, or is converted to ketone bodies such as acetoacetate, D-β-hydroxybutyrate, and acetone, used as energy fuel for several extrahepatic tissues including the brain [24]. Furthermore, the fatty acid oxidation process is regulated by the peroxisome proliferator activated receptor alpha (*PPAR*)-α. Hence, a disruption in PPAR-α or in the fatty acid oxidation leads to a reduced capacity to use the FFA energy and the development of steatosis [24]. Moreover, the activation of SREBP-1c increases malonyl CoA in the mitochondrial membrane by the inhibition of FFA β-oxidation, as it is directed towards the formation of triglycerides.

The increase in lipid production and accumulation causes hepatic steatosis [20]. However, the precise mechanisms involved in the dysregulation of fatty acid oxidation are not fully elucidated in subjects with NAFLD. Several studies conducted in rodents exposed to high amount of FFAs showed upregulated expression of *CPT 1a* [23,25], but data from another study suggested that fatty acid oxidation genes are overexpressed while *CPT 1a* and *PPAR-α* expression are decreased in 50% in human livers with NAFLD in comparison to normal livers [26].

1.2.1.4 Fatty acid export

The VLDL assembly is a complex process, which has two steps; the principal protein structure is the apolipoprotein B (apoB), and there is also the microsomal triglyceride transfer protein (MTP) located in the ER luminal that have action as lipid-transfer between membranes with several functions such as apoB translocation and folding [27]. Barrows and Parks. [28] evaluated the different sources of FFA involved in lipoprotein synthesis during the

transition from post-absorptive to fed state in healthy men. Investigators found that the principal substrate during post-absorptive conditions approximately 77% is originated from recycled adipose tissue fatty acids and around 4% from lipogenesis [28]. Also, during feeding, the adipose tissue fatty acids give up 44%, lipogenesis more up to 8%, around 15% from the chylomicron-remnant triglyceride, and approximately 10% from dietary [28]. In this context, a chronic hepatic lipid overload coming to DNL or exogenous fatty acids facilitated lipid accumulation and storage into hepatocytes or increased VLDL secretion [27]. Indeed, a state of insulin resistance induces VLDL and apoB secretion due to inhibition of the hepatic MTP expression. Hence, both conditions develop into hepatic steatosis [29]. In line with these findings, Choi et al. reviewed the relationship between insulin resistance and abnormality in VLDL assembly in hepatic steatosis, and suggested that uncoupled protein response (UPR) and ER stress are strongly associated [30]. The mechanism that UPR inhibit hepatic VLDL output ocurring in two ways: increasing co-translational degradation of apoB by the proteasome, or promoting post-ER degradation of apoB and VLDL by autophagy. In low grades of ER stress, fatty acids overload in the hepatocytes induces VLDL secretion and triglyceride storage. But, in severe ER stress the UPR is not able to reestablish the ER protein-folding homeostasis, so it initiates several apoptotic signalling pathways [30]. A study evaluated apoB-100 synthesis in seven subjects with NASH proven by liver biopsy, seven obese subjects without NASH, and seven lean healthy subjects using an isotope ^{13}C-leucine. Data showed that the absolute synthesis and metabolic clearance rates of apoB-100 were significantly lower in subjects with NASH than in only obese and lean subjects. These results indicate that lower synthesis of apoB-100 has contributed to retention of lipids within hepatocytes [31]. These results indicate that lower synthesis of apoB-100 has contributed to retention of lipids within hepatocytes [31] .

The accumulation of lipids at the liver level causes activation of Kupffer cells, factor NFkB, leading to increased expression of inflammatory cytokines such as TNF-α, IL-6, and IL-8; a disruption of adiponectin and leptin levels associated with insulin resistance; and mitochondrial dysfunction and ER stress due to excessive FFAs, causing ROS and lipid peroxidation. Hence this chronic lipotoxic state activates several pathways linked to insulin resistance, inflammation, and apoptosis that promote hepatocyte necroinflammation, fibrosis, and the progression of this disease toward liver cancer.

1.3.2. Second Hit: oxidative stress
1.3.2.1. Oxidative stress in NAFLD

The mechanism of NAFLD pathogenesis, as previously explained, could be defined by the hit's hypothesis. In the first hit, several factors such as inadequate dietary patterns, obesity,

insulin resistance and MetS induce an excessive lipid accumulation that promotes an environment of lipotoxicity, triggering to the second hit that leads to the activation of inflammatory and oxidative cascades, contributing to hepatocellular damage, impaired liver regeneration capacity, and hepatocellular death, which increases the risk of developing HCC.

Oxidative stress is a state of imbalance between the production of reactive nitrogen species (RNS) or ROS and the antioxidant molecules of the organism responsible for the detoxification of these radicals, which lead to cellular injury and damage [32]. ROS includes several molecules such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical [32]. Moreover, the production of ROS stimulates the enzymes involved in cell protection such as superoxide dismutase, glutathione peroxidase, and catalase [32]. However, in an environment of oxidative stress, the overproduction of prooxidant species saturates the antioxidant machinery. Furthermore, ROS is closely related to lipid peroxidation, which is relevant in the pathogenesis of NAFLD [32]. In this sense, during oxidative stress, the polyunsaturated fatty acids (PUFAs) are the main substrate for lipid peroxidation by ROS species [33], so this process activates several reactive products such as hexanal, 4-hydroxynonenal derived from omega 6, 4-hydroxy-2-hexenal from omega 3, and Malondialdehyde that can diffuse into intracellular or extracellular space, affecting other cells linked to pathological conditions, tissue damage, and liver failure by stimulating the hepatic stellate cells (HSC) [20,34].

1.3.2.2 Mitochondrial dysfunction

Lipotoxicity induces the progression of simple steatosis to NASH; this leads to oxidative damage promoting the activation of many cell signalling pathways related to proinflammatory cytokines production and activation of HSCs, in which mitochondrial dysfunction leads to ROS and cytokines play a pivotal role in NAFLD pathogenesis [35–37]. Mitochondrial dysfunction and structural changes have been observed in hepatocytes of NASH patients. These mitochondrial changes include a depletion in mitochondrial DNA, decreased activity of respiratory chain, and impaired mitochondrial β-oxidation [35]. The principal abnormalities of mitochondria morphology in NASH are megamitochondria containing linear crystalline inclusions, and loss of cristae and paracrystalline inclusion bodies [38].

Mitochondrial DNA damage has been reported in NASH subjects. Kawahara et al. evaluated mutations in mitochondrial DNA in 24 subjects with alcoholic hepatitis (AL-Hep), NASH, and fatty liver (FL).

NASH and AL-Hep groups showed significantly increased mutations in mitochondrial NADH dehydrogenase subunit 1 and mitochondrial Cytochrome c oxidase subunit II of the coding region in comparison to FL group. These results indicated that gene mutations in mitochondrial DNA are observed in AL-Hep and NASH subjects, which could play a key role in liver pathogenesis [36].

Another mitochondrial dysfunction is related to the respiratory chain. Some clinical studies have reported alterations in complex I, III, IV and V in NASH subjects [39].

One study directly quantified mitochondrial respiration in liver tissue in obese (OBE) insulin-resistance patients diagnosed with NAFL (OBE NAFL+), without NAFL (OBE NAFL), with NASH (OBE NASH), and lean without NAFLD (CON). Data showed that obese insulin resistance subjects diagnosed with NAFLD or no NAFLD exhibited 4.3 to 5.3 fold high maximal respiration rates than lean subjects [39]. Moreover, NASH patients presented higher mitochondrial mass, but less maximal respiration. Thus, OBE NASH showed elevated c-Jun N-terminal kinase (JNK) phosphorylation and disruption of hepatic catalase activity, important for detoxification of hydrogen peroxide. This metabolic evidence suggests that in initial stages of obesity and insulin resistance, the mitochondria suffer an adaptation considered as "hepatic mitochondrial flexibility". However, this capacity is lost in obese NASH subjects, which present lower mitochondrial respiration and proton leakage with an increase of oxidative stress as a consequence of a reduced antioxidant capacity [39].

As previously mentioned, increased fatty acid β-oxidation in steatosis leading to increased ROS induces dysfunction in the electron transport chain, contributing to an imbalance of $NADH/NAD^+$ and $FADH_2/FAD$ (flavin adenine dinucleotide), which affect the oxidative capacity of the mitochondria and overproduction of ROS. This redox disequilibrium increased the cytochrome P450 Family 2 Subfamily E Member 1 (CYP2E1) activity that is related to NASH. CYP2E1 is suppressed by insulin, but in a state of insulin resistance is overexpressed and predisposes to oxidative stress in humans and animal models [40]. Abdelmegeed et al. evaluated the CYP2E1 activity in wild-type mice with steatohepatitis induced by high-fat diet (HFD). Data revealed that WT mice HFD exhibited insulin resistance, increased CYP2E1 activity and JNK phosphorylation, malondialdehyde and hydroxyalkenals, and lipid peroxidation [41].

Wei et al. have proposed a mechanism that can explain mitochondrial abnormalities in NAFLD. This encompasses overproduction of ROS, increased TNF-α expression, and alteration of the transcription coactivator peroxisome proliferator-activated receptor-γ coactivator-1α [35]. In this context, TNF-α might promote cell death by activating the caspase cascade (proapoptotic) and NF-κB (antiapoptotic) pathways in hepatocytes [37]. Thus, TNF-α

cytotoxicity could promote damage in the mitochondrial respiratory chain at complex III, triggering an increased production of ROS and RNS [42]. On the other hand, impaired activity of the transcription coactivator peroxisome proliferator-activated receptor-γ coactivator-1α affects mitochondrial biogenesis [35].

1.3.2.3 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a dynamic organelle involved in fundamental cell processes, including production, secretion, and transport of membrane proteins, lipid biosynthesis, carbohydrate metabolism, and intracellular calcium storage. The ER is a complex system of tubular membranes that includes two continuous membranes: the nuclear envelope and the peripheral ER. An oxidative stress environment can trigger crucial changes in the ER membrane environment, which has a negative impact on ER functions and UPR activation. The UPR signalling is mediated by the protein kinase-like ER kinase, inositol-requiring enzyme 1α, and activating transcription factor 6α [43,44]. Furthermore, these UPR transmembrane sensors are involved in lipid metabolism [45]. The progression of NAFLD to NASH is thus strongly associated with ER stress and UPR activation.

UPR/ER stress has been linked to activation of inflammatory (NF-κB, JNK, ROS, TNF-α, IL-6) and insulin pathways [46], lipotoxicity and apoptotic cell death which are common features in NAFLD and obesity [45]. Pagliassotti et al. published a review, evaluating the link between ER stress and UPR and obesity and related chronic diseases manifestations [44].
This suggested that ER stress might be activated by three mechanisms: the increased levels of protein synthesis, inducing ER stress; a downregulation of the folding capacity leading to ER stress mediated by disequilibrium of calcium homeostasis between ER lumen and cytosol increasing the production of chaperones; and a reduction in the degradation of misfolded ER proteins [44]. However, the precise mechanism in progression to the pathogenesis of NAFLD is not fully defined.

ER stress might play a crucial role in steatosis by the action of lipid transcription factors [47]. Colgan et al. have evaluated the effects of ER stress on lipid metabolism. Cell lines (HeLa and MCF7) treated with ER stress-inducing agents showing that ER stress induces SREBP-2 activation leading to lipid accumulation. Moreover, cholesterol accumulation and SREBP-2 activation are mediated by site-1-serine protease and site-2-zinc metalloproteinase proteolytic networks [47].

On the other hand, several studies have documented that FFAs are implicated in the activation of UPR and liver injury [46]. Arruda et al. (2014) studied the impact of saturated fatty acids over the ER in rat hepatoma cell lines (H4IIE liver cells) and primary hepatocytes.

Data showed that palmitate promotes the activation of UPR and the markers associated with ER stress and cell death. Saturated fatty acids also induce changes in calcium transport activity that triggers hepatocytes death [48]. Zhang et al. [45] reviewed the role of ER stress in NAFLD, finding that ER stress might induce hepatocyte death thought C/EBP-homologous protein (CHOP) pathway; the induction of the JNK pathway by mediated IRE1 recruitment of protein TNF-α receptor-associated factor 2 TRAF2; and a dysregulation of calcium homeostasis signaling pathways [45].

In summary, an increased uptake of FFA induces hepatic lipotoxicity, inflammation, and oxidative stress inducing ER perturbation. This state promotes the activation of many pathways to establish the normal ER homeostasis. However, in a constant lipotoxic environment, ER dysfunction induces steatosis and hepatocyte injure.
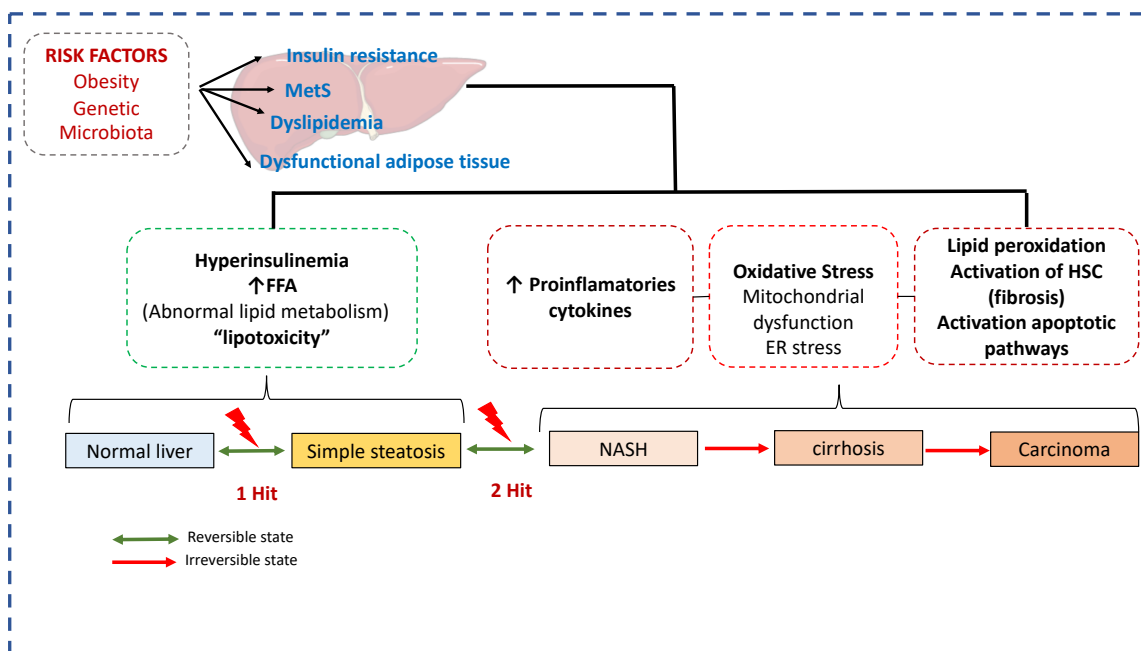
Figure 1. Pathogenesis of non-alcoholic fatty liver disease.

Pathogenesis of nonalcoholic fatty liver disease. Several risk factors mainly induce insulin resistance and dysfunctional adipose tissue. This state increases production of several proinflammatory mediators and promotes alterations in lipid metabolism, developing a state of lipotoxicity which leads to hepatic steatosis (first hit). This environment of oxidative stress induces increased ROS production, mitochondrial dysfunction, and ER stress, leading to liver damage by activation of HSC and apoptotic pathways (second hit). Abbreviations: MetS, metabolic syndrome; FFA, free fatty acid; ER, endoplasmic reticulum; HSC, hepatic stellate cell; NASH, nonalcoholic steatohepatitis.

2.   RISK FACTORS ASSOCIATED WITH NON-ALCOHOLIC FATTY LIVER DISEASE

2.1.   Ethnicity, age and sex

Several epidemiological studies based on multi-ethnic data suggested that NAFLD prevalence differs between race and ethnicity groups, while incidence is higher in Hispanics compared to non-Hispanic blacks populations [49]. Moreover, Hispanics have an increased risk to develop NASH in comparison to Blacks, once the NAFLD is developed [50]. Black adults are likely as white adults to develop obesity and T2DM, which are closely related to NAFLD pathogenesis [50]. Disparities in NAFLD prevalence could be attributable to social, economic, cultural, environmental, genetic, and other factors [49,50]. Genetic variations could explain the inter-individual differences in the risk or presence of NAFLD [49].

Genome-wide associations studies indicated that genetic variations in the genes patatin-like phospholipase domain-containing 3 and transmembrane 6 superfamily member 2 (PNPLA3) are involved in the ethnicity disparities in hepatic steatosis and NAFLD prognosis [51]. In humans, the PNPLA3 genotype is associated with hepatic fat accumulation and early age of NAFLD with pronounced effects in Hispanics [49,51]. Body fat distribution differs between sexes where men mostly accumulate adipose tissue in the abdominal area, while women in the gluteal-femoral region [52]. However, race differences in body distribution, especially in women, have been reported [49]. NAFLD occurs more often in men than women in young adulthood, but this tendency affects in the middle and early age [1,53]. The incidence of metabolic disorders has increased in early age where after menopause, estrogen levels decline triggering to essential changes in fat distribution characterized by increased VAT and ectopic fat accumulation [53]. In this context, NAFLD pathogenesis might be influenced by hormonal status.

2.2.   Obesity

Obesity is characterized by excessive body fat mass, usually accompanied by a low-grade chronic inflammation resulting in an imbalance between energy intake and expenditure [54]. However, obesity aetiology is more complicated where the interaction of economic, genetics, gut microbiota, environmental and eating behaviour are involved [54]. Obesity is a significant risk factor of several chronic illnesses, including CVD, diabetes, NAFLD, and others [54].

The rising prevalence and severity of NAFLD are closely associated with obesity [1] [55]. Pang et al. 2015 showed that subjects with higher body mass index (BMI) and waist circumference were associated with the risk of NAFLD [56].

Individuals with a BMI of <25 kg/m^2 had a low risk of high liver fat (5%) [57]. Meanwhile, the prevalence of higher liver fat was 1 in 3 in subjects with higher BMI (>30 kg/m^2) [57]. Increased adipose tissue accumulation is associated with insulin resistance, disrupted lipid metabolism and increased release of several proinflammatory mediators that promote oxidative stress and liver damage [58,59]. In a meta-analysis evaluated the impact of central obesity in the incidence of NAFLD [56]. Furthermore, other study showed that the risk of NAFLD increased 1.34-, 1.85- and 3.06-fold in individuals with higher waist circumference, BMI and waist-to-hip ratio, respectively [56].

Visceral and subcutaneous adipose tissues are the primary source of fat depots and different adipocytokines profiles [58]. In NAFLD, adipocytokine dysregulation mainly characterized by increased levels of TNF-α and leptin as well as decreased levels of adiponectin [58]. Leptin is implicated in the regulation of appetite and bodyweight [59]. Some studies evidenced that leptin levels were elevated in subjects with obesity and cirrhosis [59]. In the Dallas Heart Study, VAT has associated subjects with adipocytokine and lipid metabolism disruption (dyslipidaemia) and atherosclerosis obese phenotype compared to abdominal subcutaneous fat (SAT) [59].

2.3. Insulin resistance and type 2 diabetes mellitus

Insulin is a hormone secreted by βeta cells of the pancreatic islets of Langerhans that regulate blood glucose levels, as well as participate in the regulation of carbohydrate, lipid, and protein metabolism [60]. The main effects of insulin on skeletal and adipose in the carbohydrate metabolism, includes the increase in glucose uptake into cells by insulin-dependent via glucose transporter (GLUT) 4 and promotes glycogen synthesis and decrease the rates of glycogen breakdown [60]. In the lipid metabolism, insulin stimulates lipogenesis and decreases the rate of lipolysis in adipose tissue regulating the FFAs flux into the bloodstream [60]. Likewise, insulin stimulates FFA and triacylglycerol synthesis in tissues, increases the uptake of triacylglycerol from the blood into adipose tissue and muscle, as well as insulin decreases the rate of fatty acid oxidation in the muscle and liver [60]. This state is defined as a pathological condition characterized by an inadequate response to the insulin in peripheral targets tissues presented on skeletal muscle, liver and adipose tissue [60,61]. Insulin resistance decreases the expression of the LPL; thus, triglycerides attached to lipoproteins accumulate in the bloodstream and tissues [11]. Moreover, the activity of the hormone-sensitive lipase increases the export of FFAs to the plasma. This state leads to higher amounts of FFAs available for uptake into hepatocytes, promoting oxidation and esterification of triacylglycerol (lipid droplets) into hepatocytes, developing liver steatosis and increased risk of NAFLD [11].

T2DM is related to insulin resistance where a progressive dysfunction of the β cells resulting in insulin resistance and impaired insulin sensitivity trigger to altered glucose regulation [60,61]. T2DM increases the prevalence and severity of NAFLD [62,63]. A recent meta-analysis based on population-based observational studies, which included 22 studies showed that T2DM is associated with a 2-fold increase risk of developing severe liver disease [63]. Several methods for the estimation of insulin resistance have been proposed. At present, the glucose clamp technique is the gold standard for quantifying insulin sensitivity [64]. Nevertheless, this an expensive and time-consuming method, which is challenging to apply in large-population studies [64]. Consequently, feasible indices have been used as a practical and robust tool in epidemiological studies and clinical practice [64]. One of this marker is the homeostasis model assessment of insulin resistance (HOMA-IR) that included fasting serum glucose and insulin levels, which determinate insulin sensitivity and β-cell function [64]. Another model is the product of fasting triglyceride and glucose levels (TyG) index, which is a novel index Vascular-Metabolic CUN cohort. At an 8.84-year follow-up, investigators found an increased risk of T2DM in participants with TyG ≥8.31 [65]. Also, the TyG index is associated with metabolic features [66] and NAFLD [67,68].

2.4. Dyslipidaemias

Disturbances in lipid profile are often frequent in patients diagnosed with NAFLD [69]. increased levels of cholesterol characterize dyslipidaemia in NAFLD, higher levels of triglycerides and low-density lipoprotein cholesterol (LDL-c) as well as lower levels of high-density lipoprotein cholesterol (HDL-c) [69]. An imbalance of FFAs flux and impaired VLDL-c leading to insulin resistance is linked to risk of atherosclerosis and coronary artery disease in NAFLD [69,70]. This increase is also linked to upregulate apoB-100 particles, VLDL and triglyceride content [70]. The cholesterol ester transfer protein is an enzyme implicated in the exchange of cholesterol esters and triglycerides between VLDL, HDL, and LDL [70]. In NAFLD, the cholesterol ester transfer protein is stimulated by increased levels of VLDL trigger to higher triglyceride transfer between VLDL, LDL-c and HDL-c particles generating triglycerides rich in LDL-c and HDL-c, respectively [70]. These changes promote increased HDL-c catabolism and clearance by the kidneys and consequently, lower serum HDL-c levels [70]. Therefore, atherogenic dyslipidaemia increases the risk of CVD in NAFLD.

Oxidative stress is a hallmark in NAFLD progression [71]. Lipid peroxidation and inflammation are common features in NASH [71]. This state leads to mitochondrial dysfunction and endoplasmic reticulum stress cause structural changes in hepatocytes of NASH subjects [71–73].

Oxidized low-density lipoproteins (oxLDL) has been described as an important factor in hepatic damage [72] while impaired oxLDL exchange inside Kupffer cells triggers inflammatory and apoptosis mediators that could be involved in NASH [72]. Moreover, Musso et al. showed that postprandial VLDL accumulation was associated with oxLDL, total antioxidant status, increased ALT levels and liver steatosis in NASH individuals [73].

In a systematic review and meta-analysis, Bonci et al. demonstrated that NAFLD increased risk to present diastolic left ventricular dysfunction and changes in the cardiac structure measured by the left ventricular mass index, increased intima-thickness and arterial stiffness [74]. In agreement with these results, a population-based study revealed that NAFLD is strongly associated with abnormalities in the left ventricular structure and function in a short follow-up period [75]. Also, investigators suggested that obesity is an independent factor of these myocardial dysfunction [75]. In this sense, it is suggested that individuals diagnosed with NAFLD could have modifications in myocardial substrate metabolism trigger cardiac dysfunction and hypertrophy [76]. These alterations are potential risk factors associated with congestive heart failure [76].

2.5. Metabolic syndrome

MetS is characterized by a cluster of metabolic components such as central obesity, higher levels of fasting glucose and triglycerides, hypertension and lower levels of HDL [77]. Nowadays, several diagnostic criteria for MetS have been proposed (Table 3) [77]. Therefore, the prevalence of MetS could vary according to criteria used [77]. However, it is recognized that MetS is a crucial factor to be susceptible to chronic conditions [78]. NAFLD is mainly associated with insulin resistance, dyslipidaemia and abdominal obesity, which are the main components of MetS [1,2,78]. Likewise, it has suggested that NAFLD is the hepatic manifestation of NAFLD [5,6,78].

In a meta-analysis have assessed the risk of T2DM and MetS in individuals diagnosed with NAFLD during a 5-years follow-up [79]. NAFLD was associated with increased risk T2DM and MetS with a relative risk of 1.97 and 1.80, respectively [79]. Scientific evidence suggests that MetS could increase the risk of severe fibrosis mainly associated with insulin resistance and dyslipidaemia [71,72]. Recently, Alexander et al. evaluated primary care databases from four European countries and estimate the risk of NASH and HCC in NAFLD patient's vs matched control [80]. Individuals with NAFLD presented higher prevalence of T2DM, hypertension and obesity than control [80]. Moreover, increased hazard ratios for cirrhosis (4.73) and HCC (3.51) were found in NAFLD subjects compared to control group [80]. In the Rotterdam study, researchers found that MetS components were independently associated with NAFLD [81]

Table 3. Diagnostic criteria for metabolic syndrome (MetS)

	NCEP-ATP III (2003)	IDF (2005)	IDF-AHA/NHLBI (2009)
Definition of MetS	Minimum 3 criteria	Central obesity plus 2 criteria	Minimum 3 criteria
Glucose	≥100 mg/dL	≥110 mg/dL	≥110 mg/dL
Waist circumference†	Male >102 cm Female >88 cm	Male ≥ 94 cm Female ≥ 80 cm	Male ≥ 94 cm Female ≥ 80 cm
Hypertension	≥130/85 mmHg	≥130/85 mmHg	≥130/85 mmHg
Triglycerides	≥150 mg/dL	≥150 mg/dL	≥150 mg/dL
HDL- cholesterol	Male <40 mg/dL Female <50 mg/dL	Male <40 mg/dL Female <50 mg/dL	Male <40 mg/dL Female <50 mg/dL

Modified from Kassi et al. [77] ATP-III, National Cholesterol Education Program; IDF, International Diabetes Federation; AHA, American Heart Association/National Heart, Lung, and Blood Institute; HDL-c, high density lipoprotein cholesterol. †European population. † Waist circumference according to population and country-specific values.
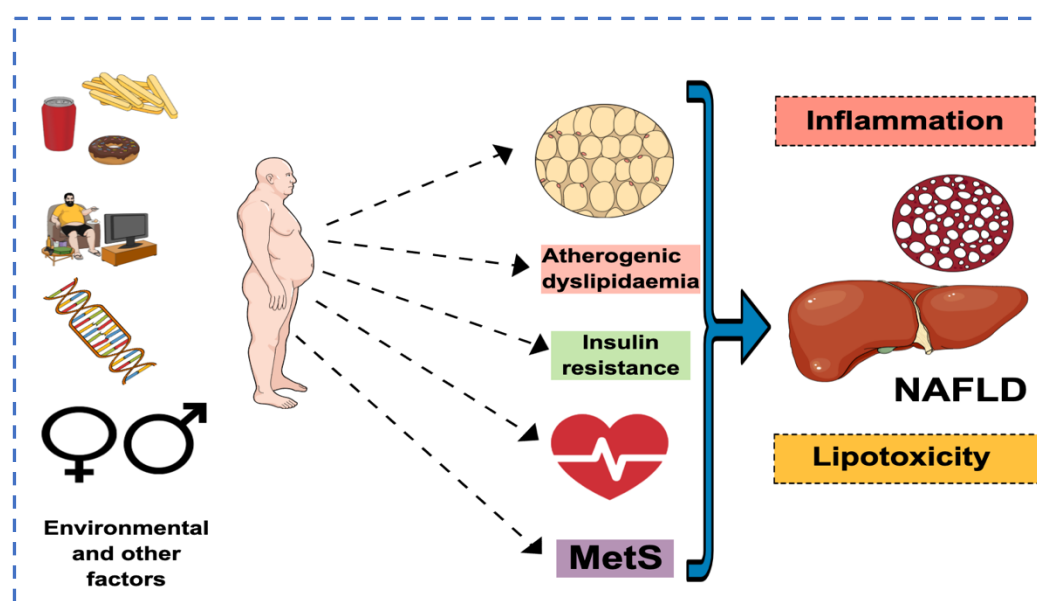
Figure 2. Risk factors associated with NAFLD development and disease progression.

Sex-age related differences, unhealthy diet, sedentary behaviours, genetic, environmental and other factors increased the risk of obesity. Central obesity is strongly associated with a disrupted adipose tissue function, atherogenic dyslipidaemia, and hepatic/peripheral insulin resistance leading to CVD and MetS. This lipotoxicity and inflammatory state develop NAFLD initiating with simple hepatic steatosis, which increased the risk of HCC in the last stages.

3.   DIAGNOSIS

Features of MetS are not only highly prevalent in individuals with NAFLD, but MetS abnormalities also increase the risk of developing NAFLD [1]. In this sense, the European clinical practice guidelines, recommending the screening in patients with suspected NAFLD by liver enzymes and/or imaging technique in the routine workup [1,2]. Liver biopsy is the most reliable procedure of hepatic lesions, but it is limited by several reasons [1,2]. Methods for the diagnosis of NAFLD are reviewed below.

3.1.   Liver biopsy

Liver biopsy is still considered the gold standard for diagnosing NAFLD and for establishing NASH (degree of liver fibrosis and liver damage) [82]. Nevertheless, this approach is an invasive procedure that could be hampered by clinical complications increasing the risk of morbidity and mortality [82]. Furthermore, sampling differences and variability in the technique, and as a result, an inaccurate diagnosis [82].
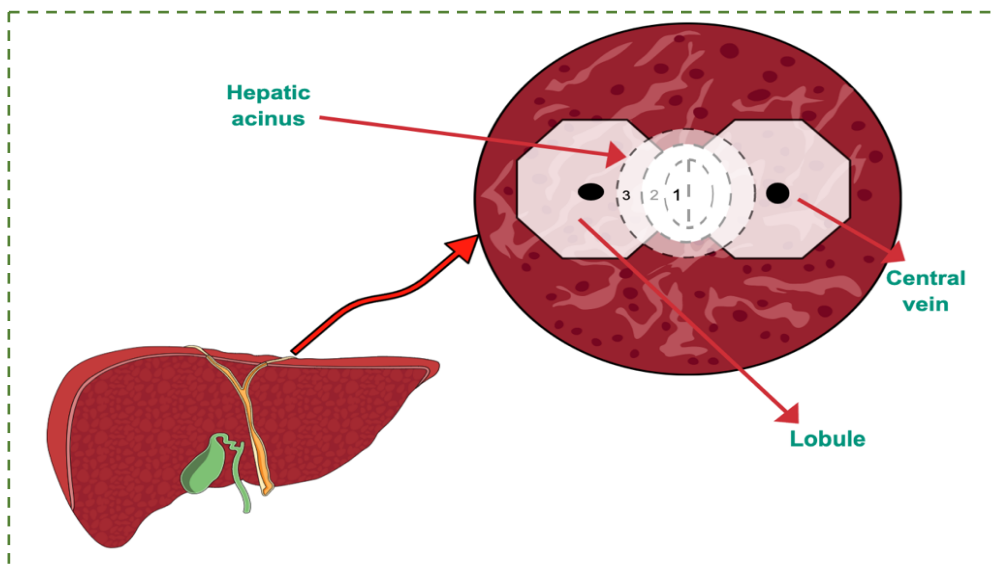
Figure 3. Liver structure. Ovalle and Nahirney [83]

The liver structure is organized into acinus consisting of two portal spaces and two centrilobular veins [83]. Liver acinus is distributed in 3 zones [83]. Zone 1 is closest to the portal space, zone 2 is between zone 1 and 3, and zone 3 is the closest to the central lobular vein. Zone 1 receives a blood flow with a higher concentration of oxygen while zone 3 hepatocytes receive a lower concentration of oxygen, susceptible to hypoxia, and zone 2 shows intermediate characteristics between zones 1 and 3 previously described [83]. Steatosis severity is determined by considering the number of hepatocytes compromised by lipid vacuoles, predominantly macrovesicular or a combination of small and large lipid vacuoles (microvesicular and macrovesicular), mostly concentrated in zone 3 [83].

3.2. Conventional laboratory markers

Liver enzymes are evaluated on routine testing used in clinical practice, where alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are usually elevated in liver diseases [2]. NAFLD could be a common cause of unexplained abnormality ALT elevations [2]. Moreover, ALT elevations have been observed in metabolic disorders [84,85]. Prati et al. showed that ALT levels <30 IU/L in men and <19 IU/L in women had a superior sensitivity (76.3%) and sensibility (88.5%) to the typical values in men (<40 IU/L) and women (<30 IU/L) [86]. This cut-off allows to include individuals with minimal or mild histological liver lesions [86]. Other laboratory test related to a liver function includes gamma-glutamyl transferase (GGT) and alkaline phosphatase [87]. Increased levels of these biomarkers also have been reported in NAFLD individuals [87].

3.3. Imaging techniques

Imaging techniques such as ultrasonography, magnetic resonance imaging (MRI), computed tomography (CT), proton magnetic resonance spectroscopy (^{1}H-MRS) and elastography are currently used in the clinical diagnoses of NAFLD [1,88]. Nonetheless, these imaging tools could not differentiate between subtypes of liver steatosis and histological stages in NAFLD and NASH, respectively [88,89]. Ultrasonography is a simple and inexpensive tool for diagnostic of NAFL. In a systematic review and meta-analysis showed that ultrasound had 84.8% of sensitivity and 93.6% of specificity for the detection of the moderate-severe fatty liver compared to liver biopsy [89]. However, in another study, ultrasonography, CT, and MRI were not able to distinguish histological differences in NASH (ballooning and fibrosis) and liver fat [90]. MRI and CT have used as noninvasive techniques in clinical trials, but the high cost limits its use [88]. The transient elastography measures the stiffness or elasticity in soft tissue by the propagation speed of elastic waves [91]. This technique could be a reliable imaging test for the diagnostic of liver fibrosis but the measure of liver stiffness in morbidly obese patients or who have ascites [91]

3.4. Non-invasive hepatic scores of NAFLD severity

3.4.1. Hepatic steatosis index (HSI)

The hepatic steatosis index (HSI) was derived from a cross-sectional case-control study involving more than 10,000 individuals diagnosed with NAFLD by ultrasonography. Values <30 had a sensitivity of 93.1% to exclude NAFLD and values >36 could detect NAFLD with a specificity of 92.4% [92]. Moreover, the HSI was associated with insulin sensitivity and β-cell function as well as it has shown an AROC of 0.79 for hepatic lipid content [93].

$$HSI = 8 \times ALT/AST \text{ ratio} + BMI \ (+2, \text{if diabetes}; +2, \text{if female})$$

3.4.2. Fatty liver index (FLI)

Bedogni et al. developed the fatty liver index (FLI) in the DIONYSOS Nutrition and Liver Study performed in two communities in northern Italy, which it considers BMI, waist circumference, triglycerides and GGT in the algorithm [94]. Investigators showed that values ≥60 had a positive likelihood ratio of 4.3 to ruled fatty liver [94]. Moreover, in a Korean cohort, the FLI was associated with a higher risk of hypertension, T2DM and MetS [95].

$$FLI = \frac{e^{0.953 \times \log(\text{triglycerides}) + 0.139 \times BMI + 0.718 \times \log(GGT) + 0.053 \times \text{waist circumference} - 15.745}}{1 + e^{0.953 \times \log(\text{triglycerides}) + 0.139 \times BMI + 0.718 \times \log(GGT) + 0.053 \times \text{waist circumference} - 15.745}} \times 100$$

3.4.3. NAFLD fibrosis score (NFS)

Investigators evaluated the NAFLD fibrosis score in patients diagnosed with NAFLD by liver biopsy. Individuals were divided into two groups to construct (n=480) and validate (n=253) the model. The AUROC for the estimation in the construct groups was 0.88%, and for the validation group was 0.82% [96]. Values of -1.455 showed a good accuracy to exclude advanced fibrosis with a negative predictive value of 93% (estimation group) and 88% (validation group). Also, 0.676 cut-offs presented a positive predictive value of 90% for the estimation group and 82% for the validation group [96]. Furthermore, the NFS could be a good predictor of liver-related events, overall mortality, metabolic diseases (T2DM and CVD) and chronic kidney disease in patients diagnosed with NAFLD by liver biopsy [97].

$$NAFLD \text{ fibrosis} = -1.675 + 0.037 \times age + 0.094 \times BMI + 1.13 \times \text{impaired fasting glucose/diabetes}(yes = 1, no = 0) + 0.99 \times AST/ALT \text{ ratio} - 0.013 \times \text{platelets}(10^9 L) - 0.66 \times \text{albumin}(g/dL)$$

3.4.4. The fibrosis-4 score (FIB-4)

Sterlin et al developed The FIB4 score. Eight hundred thirty-two individuals with virus chronic hepatitis C virus (HCV) and human immunodeficiency virus (HIV) with liver biopsy were analyzed [98]. Liver biopsy was categorized by Ishak score mild (0–1), moderate (2–3), and advanced (4–6) liver fibrosis. Patients were randomized into training set (n = 555) or a validation set (n = 277). In the validation set, a cutoff of <1.45 showed a negative predictive value to exclude advanced fibrosis of 90% with a sensitivity of 70%. Moreover, a cutoff of >3.25 had a positive predictive value of 65% and a specificity of 97% [98].

$$Age \times AST[U/L]/(platelets\ [10^9/L] \times (ALT[U/L])^{1/2})$$

3.4.5. BARD score

The BARD score was evaluated in 827 subjects with biopsy-proven NAFLD (669 participants had NASH) [99]. The model that included BMI, AST/ALT ratio and diabetes mellitus increased the risk of fibrosis in NASH by 2.4 times. Also, the BARD score exhibited a positive predictive value of 43% and a negative predictive value of 96% to detect advances in liver fibrosis in stages 3-4 [99].

1. **B**MI (IMC) ≥28 kg/m² (1 point)

2. **AR** (AST/ALT ratio) ≥ 0.8 (2 points)

3. **Diabetes** mellitus (1 point)

3.4.6. BAAT score

Ratziu et al. performed the BAAT score, which includes BMI, age, ALT, and triglycerides in the model. Investigators evaluated 93 individuals who had BMI >25 kg/m² with abnormal liver enzymes [100]. Hepatic septal fibrosis was analyzed by a liver biopsy. The analyses indicated that a score of 0 or 1 had 100% negative predictive values for the diagnosis of hepatic septal fibrosis/cirrhosis [100].

1. **B**MI ≥ 28 kg/m²

2. **A**ge ≥ 50 years

3. **A**LT ≥ 2 upper limit of normal

4. **T**G ≥ 1.7 mmol/L

3.4.7. Fibroblast growth factor 21 (FGF-21)

The fibroblast growth factor 21 (FGF-21) is a hormone commonly expressed in the adipose tissue, liver and pancreas, which is implicated in several metabolic pathways related to fatty acid oxidation and glucose uptake [101]. FGF-21 is mainly produced in the liver regulated by the PPARα pathway in response to intrahepatic fatty acids. However, in liver steatosis, this sustained activation leads to a compensatory increase expression of the FGF-21 (FGF-21 resistance) [102]. Some studies suggested that treatment with FGF-21 could modulate lipid metabolism and reduce lipid accumulation in the liver through an insulin-independent pathway and modulate NAFLD progression [101]. Increased levels of FGF-21 in individuals diagnosed with NAFLD and T2DM has been observed [101,102]. Moreover, Li et al. evaluated serum FGF-21 levels in 224 participants with NAFLD and 124 control subjects [102]. Results showed that FGF-21 levels were higher in the NAFLD group compared to control. Also, the FGF-21 was positively correlated with intrahepatic triglyceride content [102].

4. NAFLD TREATMENT: LIFESTYLE MODIFICATION

Lifestyle change, considering a healthy diet, and physical activity is the main recommended treatment for NAFLD [103]. A triple hit behavioural phenotype characterized by sedentary habits, reduced physical activity, and poor diet are associated with cardiometabolic risk factors and NAFLD [103]. There has growing evidence that lifestyle modifications may be effective in improving hepatic steatosis, serum liver enzymes, glucose control and or insulin sensitivity in subjects diagnosed with NAFLD [104]. In this context, we highlight the effectiveness of lifestyle factors on principal NAFLD components.

4.1. Dietary patterns

Nutritional status plays a crucial role in health where the diet is a modifiable risk factor for diet-related non-communicable diseases [105]. Dietary patterns are currently used in several studies to evaluate the effects of diet on metabolic profile and risk to develop chronic diseases [106]. The importance of the evaluation of dietary patterns is that not only consider nutrients but also foods encompassing the entire eating pattern [105,106].

Scientific evidence suggests that higher plant-based foods exert benefits on cardiometabolic risk factors [105,107,108]. In this sense, some eating patterns such as the Dietary Approaches to Stop Hypertension (DASH) and the Mediterranean diet (MedDiet) are currently recommended for healthy promotion [105].

The DASH diet elaborated by the National Institute of health with the objective to give specific dietary advice for treating hypertension is characterized by the intake of vegetables, fruits, and whole grains. Also, it is rich in potassium, calcium, magnesium, and protein and lower in sodium and saturated and trans-fat [107]. The DASH diet has been studied in many epidemiological and clinical trials indicating that it is associated with blood pressure reductions, decreased glucose, triglycerides and LDL-c as well as reduced CVD incidence [107] [108].

4.1.1. Mediterranean diet (MedDiet)

Unhealthy dietary patterns and sedentary behaviours have a negative impact over health outcomes driving to an increased risk to develop non-communicable diseases [109]. Actually, diet and physical activity are key factors in the NAFLD pathogenesis/progression since most individuals with this pathology show altered glucose, lipid and liver profiles leading to an increased risk to develop MetS [1,2,5,110,111]. The MedDiet pattern is a traditional diet style commonly found in the Mediterranean regions characterized by high intake of (a) fruits, vegetables, nuts and legumes; (b)whole-grain cereals; (c) olive oil as a principal source of fat; (d) moderate and high intake of fish; (e) low-moderate consumption of poultry and dairy products; (f) low intake of red meats and processed meat products; and (g) moderate intake of red wine [112]. Several clinical and epidemiological studies suggest that MedDiet might improve the principal features related to NAFLD [110,111,113] The PREDIMED study (Prevención con dieta Mediterránea) tested the hypothesis that the MeDiet supplemented with extra virgin olive oil (EVOO), or nuts would be superior to the low-fat diet (LFD) for CVD protection in individuals at high risk for CVD [114]. This study was the first randomized control trial where demonstrated that MedDiet is a suitable dietary pattern in the prevention of CVD, T2DM incidence and MetS status in individuals at high risk [115].

The effects of MedDiet on CVD have been studied in many epidemiological and randomized clinical trials [114,116–118]. Briefly, in a meta-analysis considering cohort prospective studies showed that MedDiet was associated with a significant reduction of CVD incidence or mortality [RRR= 0.90; 95% CI: 0.87, 0.93)] and overall mortality (RRR= 0.92; 95% CI: 0.90, 0.94) [116]. Furthermore, Esposito et al. evaluated the long-term effects of low-carbohydrate MedDiet and low-fat diet in subjects with T2DM [117]. Results revealed that low-carbohydrate MedDiet could reduce haemoglobin A1c level (HbA1c) and HDL-c levels, blood pressure and postpone the introduction of diabetes medication by approximate two years [117]. In another study, Krombout et al. analyzed food groups, macronutrients, eating

patterns and their effects in long-term coronary heart disease (CHD) in seven Countries (12,763 men aged 40–59 years) [118]. Investigators found that adherence to MedDiet and some of their components such as cereals, vegetables and legumes were inversely associated with CHD mortality [118]. Also, higher intake of harmful dietary components (saturated fatty acids, whole milk, processed meats, confectionary preparation and sweet products) were positively associated with CHD mortality rates [118]. Adherence to the MedDiet seems to be effective in glycemic control and preventing T2DM [119-123]. In a prospective cohort study, investigators showed that subjects with high adherence to MedDiet presented 83% reduced risk of T2DM [119]. Additionally, a 2-unit increase in the MedDiet score was associated with a 35% reduced risk to develop T2DM [119]. Moreover, in the PREDIMED study, at four years follow-up, the MedDiet was associated with a reduction of 52% of T2DM incidence [120]. Furthermore, participants supplemented with MedDiet (EVOO or nuts) showed lower levels of glucose, insulin and HOMA-IR compared to individuals of the LFD group [121]. Likewise, in the European Prospective Investigation into Cancer and Nutrition (EPIC) study indicated that individuals with high adherence to the MedDiet showed a 12% reduced risk of T2DM [122]. Also, this association was attenuated after excluded olive oil, alcohol and meat suggesting their importance of these components in the results above-mentioned [122]. In a sub-study from PREDIMED were evaluated 191 subjects revealed that MedDiet+EVOO, MedDiet+nut and LFD could significantly decrease waist circumference but, only MedDiet groups had a significant reduction of body weight. Also, MedDiet groups improved glucose parameters as LFD [123].

4.1.2. Mediterranean diet in non-alcoholic fatty liver disease

The MedDiet has proposed as an appropriate option for NAFLD management [110,111,124,125]. The beneficial effects of MedDiet may be due mostly for the higher consumption of monounsaturated fatty acids (MUFAs), PUFAs, fibres and bioactive compounds [113,124,126].

Dietary intake of MUFAs and PUFAs have been associated with positive effects on liver-related outcomes [110,126,127]. Fish and seafood contain omega-3 fatty acids, eicosapentaenoic acid, and docosahexaenoic acid with positive effects on NAFLD [109]. Recently, a study indicated that fish intake was associated with lower levels of ferritin in individuals diagnosed with NAFLD [128]. Moreover, a meta-analysis demonstrated that the supplementation of n-3 PUFA had reduced levels of ALT, AST and triglycerides levels and liver steatosis [129]. Another essential component of the MedDiet is olive oil, which contains around 70-80% of MUFAs [109]. Shidfar et al. showed that a low-calorie diet supplemented

with EVOO could have significant reductions in ALT and AST concentrations in NAFLD subjects who were overweight [130]. Besides, a clinical study found that isoenergetic diet enriched in MUFAs, improving steatosis by positive changes in hepatic β-oxidation of FFAs [131].

Disturbances in gut microbiota could be influence NAFLD pathogenesis and severity [132]. A large body of evidence revealed that legumes, fruits, vegetables and whole grains provide beneficial effects on glucose-insulin homeostasis, inflammation, oxidative stress and NAFLD risk factors [111,125,133,134]. In a prospective cohort study, data revealed that higher fruit, vegetable and legume intake was inversely associated with a lower risk of CVD, non-cardiovascular, and total mortality [135]. Legumes include beans, lentils, soybeans, dried peas, chickpeas. Many studies suggested that some polyphenolic compounds such as isoflavones, genistein and daidzein due to their low glycemic index could have protective effects on lipid and glucose profile [136]. Moreover, Li et al. reported that the highest category of legume intake compared with the lowest, presenting a relative risk (95% CI) of 0.96 (0.86–1.06) for CVD mortality and 0.93 (0.87–0.99) for all-cause mortality [137]. Increased risk and prevalence of T2DM and CVD has observed among subjects diagnosed with NAFLD [1,2,5].

MedDiet polyphenol-rich foods such as red wine, grapes, berries contain resveratrol that could ameliorate inflammation, as well as has regulatory properties on cardiometabolic and liver status [138,139]. Resveratrol has shown lowering effect on insulin, glucose and metabolic markers [139]. Likewise, it could interact into potential targets responsible for endothelial and vascular function [140]. The dietary MedDiet pattern considered the low-to-moderate consumption of red wine. However, the consumption of red wine intake in NAFLD individuals is still poorly understood [141]. In NAFLD, the majority of data related to resveratrol effectiveness have obtained from cell and animal studies [138]. Theodotou et al. concluded that resveratrol supplementation could reduce liver fat and serum liver enzymes in NAFLD subjects [142]. Nevertheless, these findings are inconsistent with those of other studies, in which resveratrol did not has significant effects on histological parameters and NAFLD features [140,143]. Some studies assessing the association between MedDiet and resveratrol have presented on table 4.

Table 4. The association between Mediterranean diet and NAFLD

Reference	N	NAFLD assessment	Design	Participants	Intervention	Outcomes
Peréz-Guisado et al. [144]	14	US and liver enzymes	Prospective	Overweight men individuals with MetS and NAFLD	Spanish Ketogenic Mediterranean diet (SKMD) that included virgin olive oil and omega-3 fatty acids (fish), lower red wine intake, green vegetables and salads (carbohydrates), and proteins (fish) 12 weeks	SKMD showed significant improvements in anthropometric (weight, BMI and waist circumference), liver (ALT, AST, steatosis degree), and biochemical variables (LDL-c, triglycerides, glucose and HDL-c).
Ryan et al. [145]	12	Liver biopsy	RCT	Non-diabetic individuals with NAFLD	MedDiet vs standard low-fat-high carbohydrate diet (LF/HCD) (6 weeks)	Significant reduction of steatosis and HOMA-IR in the MedDiet group compared to LF/HCD. No differences in weight loss, GGT and ALT levels.
Kontogianni et al. [146]	73	US and/or liver biopsy and liver enzymes	Case-control study	NAFLD= 73 Healthy match control= 58	Adherence to MedDiet	Adherence to MedDiet was negatively correlated with ALT, insulin levels, HOMA-IR and severity of steatosis. Also, MedDiet was positive correlate with adiponectin levels.

Reference	N	NAFLD assessment	Design	Participants	Intervention	Outcomes
Chang et al. [147]	797	1H-MRS	Cross-sectional	797 (220 individuals diagnosed with NAFLD)	Adherence to diet quality index-international (DQI-I) or MedDiet score	Adherence to DQI-I and MedDiet was negatively correlated with intrahepatic triglyceride content.
Trovato et al. [148]	1,199	US	Cross-sectional	NAFLD group= 532 Non-NAFLD= 667	Adherence to MedDiet	Poor adherence to MedDiet was a strong predictor of NAFLD independently from overweight.
Papamiltiadous et al. [149]	94	US and/or liver biopsy	RCT	Adults diagnosed with NAFLD+ HOMA-IR>2 and at least one elevated serum ALT levels	MedDiet vs LFD (3 months)	MedDiet revealed beneficial effects on liver fat and insulin sensitivity independent of weight loss.

Reference	N	NAFLD assessment	Design	Participants	Intervention	Outcomes
Propezi et al. [150]	51	^{1}H-MRS	RCT	Adults diagnosed with NAFLD	MedDiet= 26 LFD= 25 (12 weeks)	Both diets showed a significant reduction in hepatic fat content, BMI, waist circumference and ALT levels. Within-groups have observed improvements in cholesterol, triglycerides, HbA1c and Framingham risk score were observed only in the MedDiet group.
Katsagoni et al. [151]	63	US and/or liver biopsy	RCT	Overweight /obese individuals	MedDiet group= 21 MedDiet+lifestyle (MLG)= 21 Control group= 21	50% reduction of ALT levels and liver stiffness in MLG compared to control group.
					MedDiet group MedDiet + counselling sessions	MedDiet group has improved liver stiffness than control.
					MLG group MedDiet + counselling session + moderate-vigorous physical activity ≥ 30 min/day	MedDiet and MLG presented a significant weight reduction vs control group.

Abbreviations: MedDiet, Mediterranean diet; US, ultrasonography; RCT, randomized control trial; SKMD, Spanish ketogenic Mediterranean diet; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; LF/HCD, low-fat high-carbohydrate diet; HOMA-IR, homeostasis model assessment of insulin resistance; ^{1}H-MRS, proton magnetic resonance spectroscopy; DQI- I, Diet Quality Index- International; LFD, low-fat diet; HbA1c, haemoglobin A1c level; MLG, Mediterranean lifestyle group.

4.1.3. Polyphenols

Polyphenols constitute a wide variety of compounds with antioxidants effects, which are present in plant-based foods (fruits, vegetables, cereals, legumes, herbs, spices), chocolate and beverages (tea, coffee, wine) [152]. Polyphenols can be classified according to the number of phenols rings and of the structural elements that bind these rings to one another as flavonoids, phenolic acids, stilbenes and lignans (Figure 4) [152]. Flavonoids have a standard structure consisting of 2 aromatic rings, which are bound together by 3-C atoms that form an oxygenated heterocycle and are divided into six subclasses: flavones, flavanols, flavonols, anthocyanidins, flavanones and isoflavones [152]. Phenolic acids are categorized as derivatives of benzoic acid and derivatives of cinnamic acid [152,153]. Stilbenes have two aromatic rings bound together by an ethylene or ethene bridge in *cis* and *trans*-configuration [153]. Lignans are habitually found in fibre rich-plants and characterized by dibenzylbutane structure [154].

Several studies involving animal, human and cell lines support the beneficial effects of polyphenols in CVD, T2DM, obesity, neurogenerative disease, ageing and cancer [155]. Healthy dietary patterns such as MedDiet has associated with decreased risk of non-communicable diseases and improvements in metabolic profile due to higher consumption of fruits, vegetables, olive oil, red wine, legumes, whole-grains that have high contents of polyphenols [110–112].
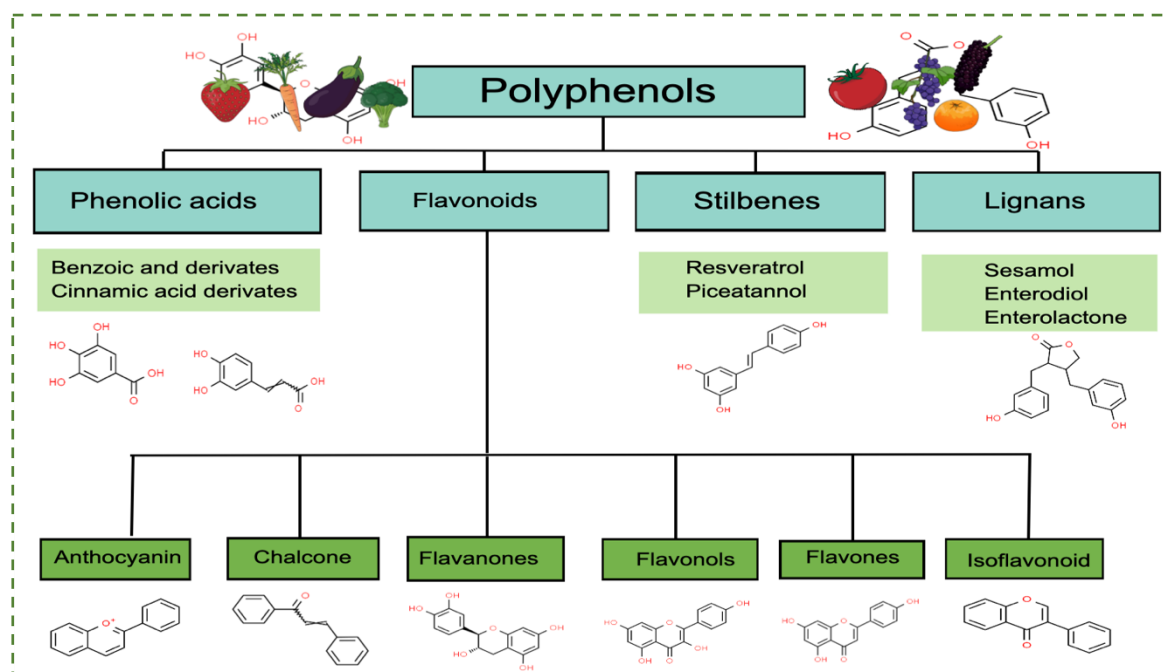
Figure 4. Polyphenol classification. Modified from Manach et al. [152] and Kumar et al. [153]

4.1.3.1. Resveratrol

Resveratrol (3,4',5 trihydoxystilbene) belongs to the stilbene family, which has 2 phenol rings bound to each other by an ethylene bridge [156]. It is presented in two isomeric forms, *cis*-and *trans*-resveratrol as well as exist as glycosylated form (piceid) [156]. Resveratrol is present in red wine, grapes, berries, peanut, pistachios, soy, itadori tea [157] and its produced in plants in response to injury, environmental stress, microbial infection or UV radiation [156] [158]. Resveratrol is synthesised by phenylpropanoid pathway, involving phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase amino acids producing cinnamic acid and 4-coumaric acid, respectively [156,158]. The cinnamic acid is further hydroxylated by the cinnamate-4-hydroxylase and converted to 4-coumaric acid. Later, the 4-coumaric acid is changed into 4-coumaroyl-CoA by the action of 4-coumaroyl-CoA ligase generating an active intermediate [156,158]. Lastly, three molecules of malonyl-CoA with 4-coumaroyl-CoA are condensed by the stilbene synthase, and its cyclisation leads to the production of resveratrol [156,158].

4.1.3.2 Human metabolism and bioavailability of resveratrol

After ingestion, resveratrol was absorbed in the intestinal enterocyte and suffered a glucuronidation and sulfation process [159]. The glucuronidation of the resveratrol is principally catalyzed by the UDP-glucuronosyltransferase (UGT), UGT1A1, UGT1A9 and UGT1A10 [159,160]. Meanwhile, the sulfation is mainly catalyzed by the sulfotransferase (SULT) SULT1A1 [159]. Conjugates resveratrol efflux via the multidrug resistance protein (MRP) 2 and breast cancer resistance protein (BCRP) located in the apical membrane and through the basolateral membrane via the MRP3 [159]. Resveratrol metabolites exit the apical membrane and go forward to the large intestine where are transformed by the gut microbiota into dihydroresveratrol (DHR), lunularin and 3,4'-dihydroxy-trans-stilbene [159]. Subsequently, resveratrol metabolites enter in the enterohepatic circulation into the liver, and they are further glucuronidated or sulfated (phase II metabolism of resveratrol) [160]. In the bloodstream, resveratrol conjugates are bound to the surface of albumin and lipoproteins (LDL) for the distribution to the target peripheral tissues followed by the elimination in the urine and faeces [159] (Figure 5).

A study has shown that *trans*-resveratrol-3-sulfates, *trans*-resveratrol-disulfates and *trans*-resveratrol-diglucuronides were bound to proteins in 33.9, 43.6 and 46%, respectively [161]. Also, in a human study, Urpí-Sardà et al. revealed that LDL was identified in *trans*-resveratrol-3-O-glucuronide, *cis*-resveratrol-3-O-glucuronide, *cis*-resveratrol-3-O-glucoside and in free trans-resveratrol after wine intake [162].

Resveratrol is highly metabolized leading to the production of conjugated sulfates and glucuronides metabolites that have shown biological activity in many target organs [139,159]. Conjugated resveratrol metabolites were identified and quantified by liquid chromatography–mass spectrometry (LC–MS) techniques, followed by mass spectrometry [139,159]. Also, their analysis needs the development of robust and accurate analytical methods [139,159,163]. Clinical studies in human have found that the primary resveratrol metabolites of urine are *cis*-resveratrol-4'-sulfate, *cis*-resveratrol- 3-O-glucuronide, and *cis*-resveratrol-4'-O-glucuronide, *trans*-resveratrol-3-O-sulfate [139,159,163]. Moreover, Rotches-Ribalta et al. validated a method for identification and quantification of resveratrol metabolites after the moderate consumption of red wine or dealcoholized red wine in participants at high CVD risk [163]. Authors were found almost 15 resveratrol metabolites after 28 days of red or dealcoholized wine intake. These resveratrol compounds included metabolites previously mentioned, pieced and gut microbiota metabolites [163].
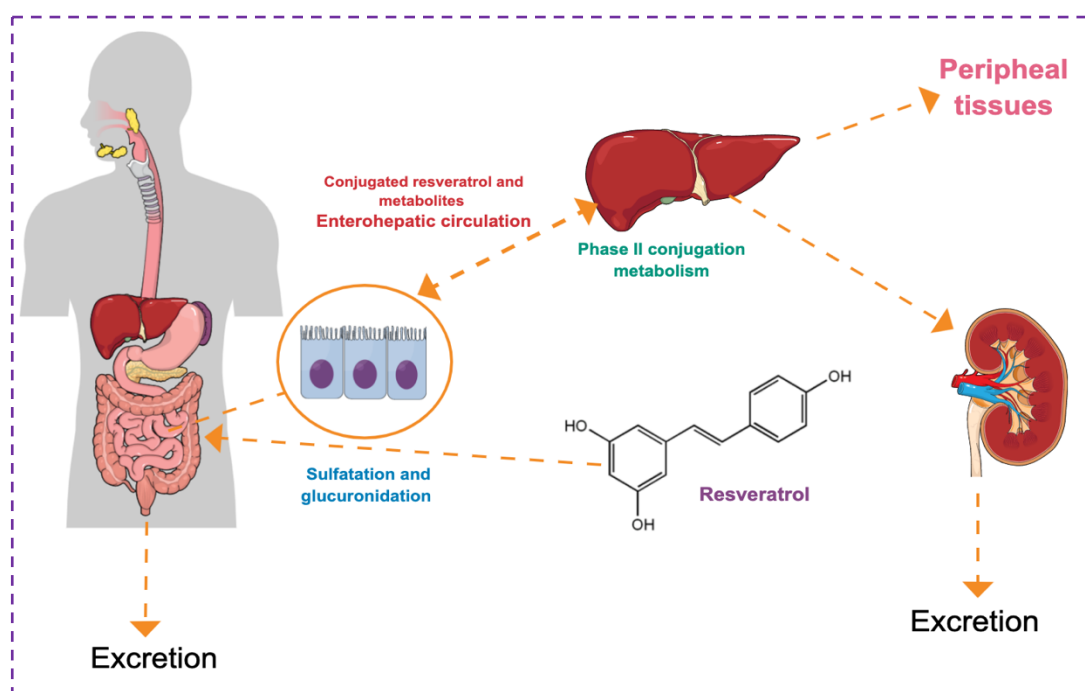
Figure 5. Resveratrol metabolism. Modified from Springer et al. [159]
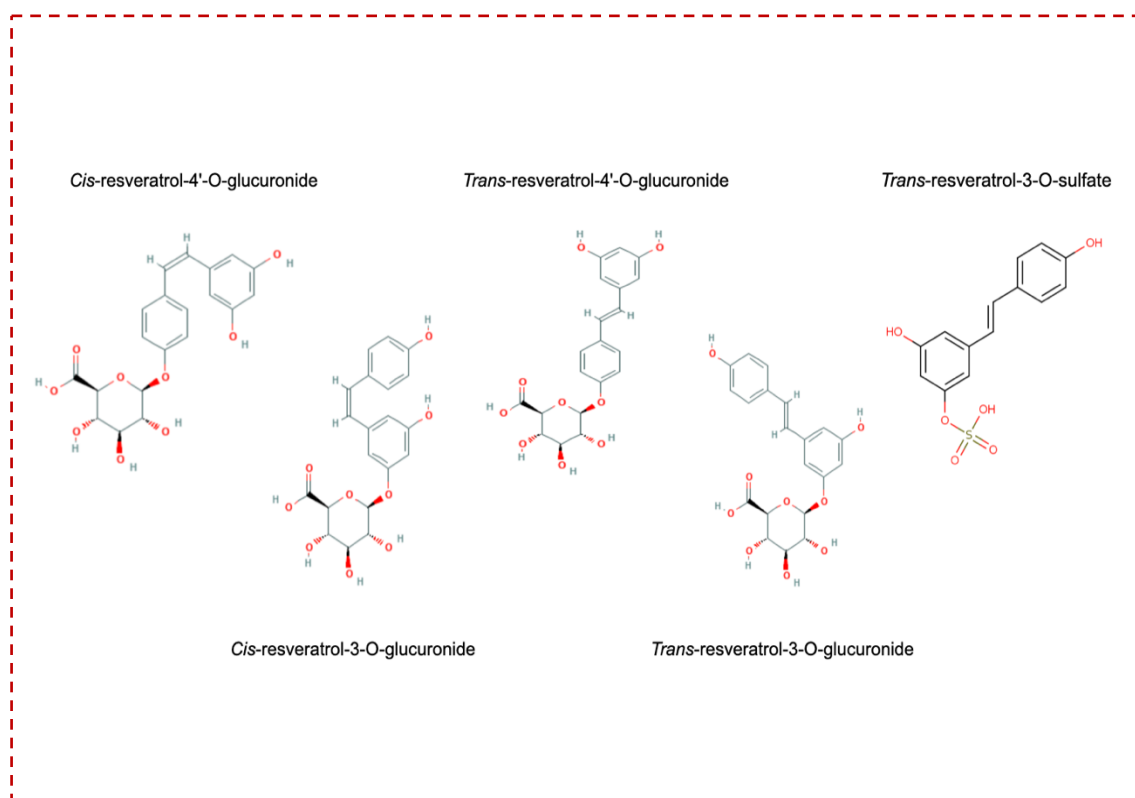
Figure 6. Resveratrol conjugated metabolites

4.1.3.3. Resveratrol in health status

The role of resveratrol in CVD has been reported in several studies [156,164,165]. The beneficial effects associated with resveratrol intake includes improvements in vasodilation, platelet aggregation, inflammation, apoptosis, oxidative stress and lipid profile [156,164]. The nitric oxide (NO) has a significant role in CVD. The NO regulates cardiovascular function involving the regulation of vascular smooth muscle cells, angiogenesis, and decrease platelet aggregation and thrombosis [166]. In this sense, resveratrol might upregulate endothelial NO synthetase, and consequent perturbations related to NO pathways [156,164]. Moreover, resveratrol could be improved gut barrier inhibiting the synthesis of trimethylamine and further formation of trimethylamine-N-oxide (TMAO) that is closely related with increased risk of atherosclerosis and other CVDs [167]. Resveratrol might suppress the mRNA expression levels of endothelial adhesion molecules (ICAM-1 and VCAM-1) attributed to the nuclear factor (NF-κB) pathway [168]. Also, it could regulate hepatic LDL receptors expression by the activation of the SREBPs in hepatocytes in vitro [169] and regulate the ATP-binding cassette transporter A1 protein expression, which mediates the cholesterol efflux [165].

Several studies in animal models, in vitro and humans, suggested that resveratrol can lead to glycemic control, increasing the expression of GLUT4, which is related to the transport of glucose in the muscles [156,170]. Moreover, resveratrol could be involved in the improvements of insulin sensitivity by the modulation of the Akt pathways, increasing SIRT-1 (involved in caloric restriction) and the AMPK expression (metabolic fuel sensory kinase) [170]. In a randomized control trial, 66 participants diagnosed with T2DM received supplementation of resveratrol (1 g/d for 45 days). Resveratrol group showed reduced levels of glucose, HbA1c, insulin, HDL, and decreased systolic blood pressure [171]. Besides, Bhatt et al. evaluated the effects of resveratrol supplementation (250 mg/d for 3 months) in parameters related with glycemic control in individuals with T2DM (control= 29, intervention= 28). Intervention group presented significant improvements in HbA1c, systolic blood pressure and total cholesterol [172].

4.1.3.4. Resveratrol in non-alcoholic fatty liver disease

NAFLD includes several complex mechanisms leading to metabolic risk factors, which have a pivotal role in NAFLD pathogenesis [1-3,71]. Resveratrol could be implicated in the regulation of many pathways related to energy homeostasis, inflammation and oxidative stress suggesting that resveratrol may be useful in NAFLD prevention and treatment [113,156,165,167,170]. It has suggested that resveratrol could be involved in the regulation of energy metabolism [156]. Also, in the improvements on lipid metabolism, mediated by the regulation of the mitochondrial β-oxidation [156]. Resveratrol has shown potential modulation in the gut microbiota composition [167]. Therefore, stimulating favourable microbial conditions. Metabolites of resveratrol might have an effect on the production of satiety hormones and improve the intestinal barrier function [167]. This point is essential to highlight, considering that gut microbiota dysbiosis is closely related to low-grade inflammation, and abnormal immune response [132]. It claimed that dysfunction of the intestinal microbiome could be implicated in NAFLD development [132]. However, fewer well-design clinical studies have studied the effects of resveratrol on NAFLD outcomes (Table 5).

Table 5. Effects of resveratrol on NAFLD

Reference	N	NAFLD assessment	Design	Participants	Intervention	Outcomes
Faghihzadeh et al. [173]	50	US and higher ALT levels	RCT	Adults diagnosed with NAFLD	500 mg/d (12 weeks) Resveratrol= 25 control= 25 Both groups received recommendations of energy-balanced diet and physical activity	Supplementation with resveratrol reduced levels of ALT and steatosis. However, resveratrol did not affect anthropometric parameters, blood pressure, insulin resistance markers and lipid profile.
Cachay et al. [174]	20	US	RCT	Men with overweight/obesity diagnosed with NAFLD	3000 mg/d (8 weeks) Resveratrol= 10 Placebo= 10	No significant changes were found in anthropometric variables, blood pressure, resting metabolic rate, insulin resistance markers, steatosis and abdominal fat.

Continue

Reference	N	NAFLD assessment	Design	Participants	Intervention	Outcomes
Chen et al. [175]	60	US	RCT	Adults diagnosed with NAFLD	600 mg/d (3 months) Resveratrol= 30 control= 30	Resveratrol group exhibited decreased levels of ALT, AST, glucose, LDL-c, total cholesterol and HOMA-IR compared to control group. No changes in hepatic steatosis. Supplementation with resveratrol showed improvements in inflammatory and liver damage biomarkers.
Asghari et al. [176]	60	US	RCT	Individuals with overweight/obesity diagnosed with NAFLD	600 mg/d (12 weeks) Resveratrol= 30 Placebo= 30	No effect on liver serum enzymes, serum malondialdehyde, ox-LDL and TAC were found.

Abbreviations: US, ultrasonography; RCT, randomized control trial; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL-c, low-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; oxidized low-density lipoprotein, ox-LDL; total antioxidant capacity, TAC.

4.2. Physical activity and exercise

A sedentary lifestyle increases the risk of developing chronic diseases and premature death [177]. For example, it has shown that sedentary behaviours (spending time watching television or sitting times) were associated with an increased risk of fatty liver [178,179]. Moreover, Scientific evidence confirms that physical activity has beneficial effects on glucose homeostasis, lipid profile, the incidence of some types of cancer, bone health, physiological well-being, CVD and others [177]. Exercise increases energy expenditure and promotes weight loss, which is one of the principal goals in NAFLD management [1]. The current clinical guidelines for the management of NAFLD do not provide specific recommendations for physical activity in this population (the type of exercise, level of intensity, or duration), but recommend lifestyle changes focus on physical activity and healthy dietary patterns [1].

Leisure-time physical activity includes structured exercises, sports and household tasks during free time that is not associated with regular occupation or transportation activities [180]. Increased moderate-vigorous physical activity with ≥ 4 metabolic equivalent task (MET) has reported a reduced risk of all-cause mortality [181]. Aerobic and resistance exercises are related to improvements in body composition, muscle strength, and exercises include activities such as walking, cycling, and swimming, which increase the peak of oxygen consumption and confers beneficial cardiovascular adaptations [182]. Meanwhile, in resistance exercises, muscles increase their strength and endurance, promoting neuromuscular adaptations [182]. A meta-analysis of 18 randomized clinical trials revealed that moderate-intensity physical activity considering aerobic and resistance exercises could have beneficial effects in individuals with NAFLD [183]. This is supported by a study that includes 3,718 in NAFLD subjects [184]. Investigators showed an inverse association between diverse physical activity types independently of VAT and insulin resistance [184]. Moreover, a clinical trial evaluated the impact of aerobic or high-intensity interval exercise on intrahepatic triglycerides and VAT in diabetic obese patients with NAFLD [185]. Results revealed a reduction of intrahepatic fat and VAT in both groups [185].

On the other hand, clinical evidence suggested that increased physical activity could have significantly beneficial effects on NAFLD features, independent of weight loss [186,187] Lee et al. studied the effects of aerobic and resistance exercise without caloric restriction on central adiposity and insulin sensitivity in obese adolescent boys [186]. Investigators showed that both type of exercise, promoting significant reductions in liver steatosis, total and VAT amounts [186]. Furthermore, it has been observed that resistance exercise exhibited increased insulin sensitivity and fat oxidation during submaximal exercise as well as the reduction of intrahepatic lipid independently of weight reductions in NAFLD participants [187]. Some physical activity studies in individuals diagnosed with NAFLD were summarized in Table 6.

Table 6. Physical activity interventions in individuals diagnosed with NAFLD

Reference	N	NAFLD assessment	Design	Participants	Intervention	Results
Gerber et al. [188]	3056	FLI	Cross-sectional	Individuals of NHANES 2003-2006 NAFLD (n=1263) Non-NAFLD (n=1793)	Accelerometer (7 days)	NAFLD individuals spent less time on physical activity at all levels (light, moderate and vigorous). NAFLD individuals with T2DM were in the lowest quartile of physical activity.
Pugh et al. [189]	13	US and liver enzymes	RCT	Adults with NAFLD Exercise group (n=7) Control group (n=6)	Moderate aerobic exercise (16 weeks)	Exercise improved cutaneous microvascular nitric oxide function. Also, ALT and AST levels decreased significantly in the exercise group. However, no changes in liver fat content.
Sullivan et al. [190]	18	^{1}H-MRS	RCT	Obese adults with NAFLD	Moderate aerobic exercise (16 weeks)	Exercise decreased intrahepatic triglyceride content. No changes in VLDL-TG and VLDL-apoB-100 secretion rates.

Reference	N	NAFLD assessment	Design	Participants	Intervention	Results
Zelber-Zaji et al. [191]	82	US	RCT	Adults with NAFLD Intervention (n=44) Control (n=38)	Resistance (3 months)	The exercise group showed a higher reduction of body fat (total, trunk and android), serum ferritin and total cholesterol.
Pugh et al. [192]	74	US and liver enzymes	RCT	Obese adults with NAFLD NAFLD group (n=34) Control group (n=20)	Moderate aerobic exercise (16 weeks)	Exercise improved flow-mediated dilatation and the peak oxygen uptake.
Cuthbertson et al. [193]	69	MRI	RCT	Adults with NAFLD Intervention group (n=38) Counselling group (n=11)	Aerobic exercise (16 weeks)	Intervention group exhibited a significant reduction in liver fat compared to counselling group but no changes in hepatic glucose production were found.
Shojaee-Moradie et al. [194]	27	US or liver biopsy	RCT	Male adults with NAFLD Intervention (n=15) Control (n=12)	Aerobic + resistance exercise (16 weeks)	Exercise increased VLDL-TG, apoB and VLDL-apoB production rate but serum triglycerides were not changed.

Abbreviations: FLI, fatty liver index; NHANES, National Health and Nutrition Examination Survey; T2DM, type 2 diabetes mellitus; US, ultrasonography; RCT, randomized control trial; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ^{1}H-MRS; proton magnetic resonance spectroscopy; MRI, magnetic resonance imaging; VLDL-TG, very-low-density lipoprotein triglyceride; very-low-density lipoprotein- apoprotein B.

II. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

1.   Hypotheses

MetS is defined by a cluster of clinical conditions, including abdominal obesity, hypertension, atherogenic dyslipidaemia, and insulin resistance, which shares common pathophysiological mechanisms with NAFLD. Indeed, NAFLD involves metabolic derangements where sex differences play a pivotal role in morbid pathogenesis and disease progression. In this context, we hypothesized if there are sex interactions on key body composition variables such as VAT, the TyG index, and non-invasive hepatic markers, which could underlie a different predisposition risk for developing NAFLD in older participants with MetS and high cardiovascular risk.

In this study, it was also hypothesized if different lifestyle behaviours including adherence to healthy eating patterns, dietary foods and specific nutritional components, as well as physical activity could provide distinct NAFLD development susceptibility in individuals with MetS at high cardiovascular risk.

2.   Objectives

2.1.   General objective

The main objective of this thesis was to investigate the influence of individual metabolic traits, the phenotype and lifestyle factors mainly focusing on adherence to the MedDiet pattern, specific food components, and physical activity on the risk of NAFLD development in subjects with MetS.

2.2.   Specific objectives

- To investigate sex differences in NAFLD risk factors in elderly overweight/obese individuals with MetS (Chapter 1).
- To explore the potential association of VAT and TyG index with the risk of NAFLD in overweight/obese subjects diagnosed with MetS (Chapter 2).
- To evaluate the relationship between lifestyle factors (adherence to the Mediterranean diet, dietary compounds, and physical activity) and the risk of NAFLD (Chapter 3).
- To assess the associations between some urinary resveratrol metabolites, cardiometabolic, and liver markers in subjects diagnosed with MetS (Chapter 4)

III. SUBJECTS AND METHODS

SUBJECTS AND METHODS

1.   The overview of the PREDIMED-PLUS study (PREvención con DIeta MEDiterránea)

The present research is an ancillary cross-sectional study of baseline data from the PREDIMED-Plus trial (PREvención con DIeta MEDiterránea). The PREDIMED-Plus study is a multicenter, randomized, parallel-group lifestyle intervention (Figure 7) designed to evaluate the effect on CVD events of an intensive lifestyle intervention based on energy-restricted traditional MedDiet, physical activity promotion and behavioural support (intervention group) compared with an unrestricted energy MedDiet without physical activity recommendations or specific goals for weight loss (control group) (https://www.predimedplus.com/) [195,196]. This study was registered in 2014 at the International Standard Randomized Controlled Trial with a number 89898870 (http://www.isrctn.com/ISRCTN89898870) [195,196].
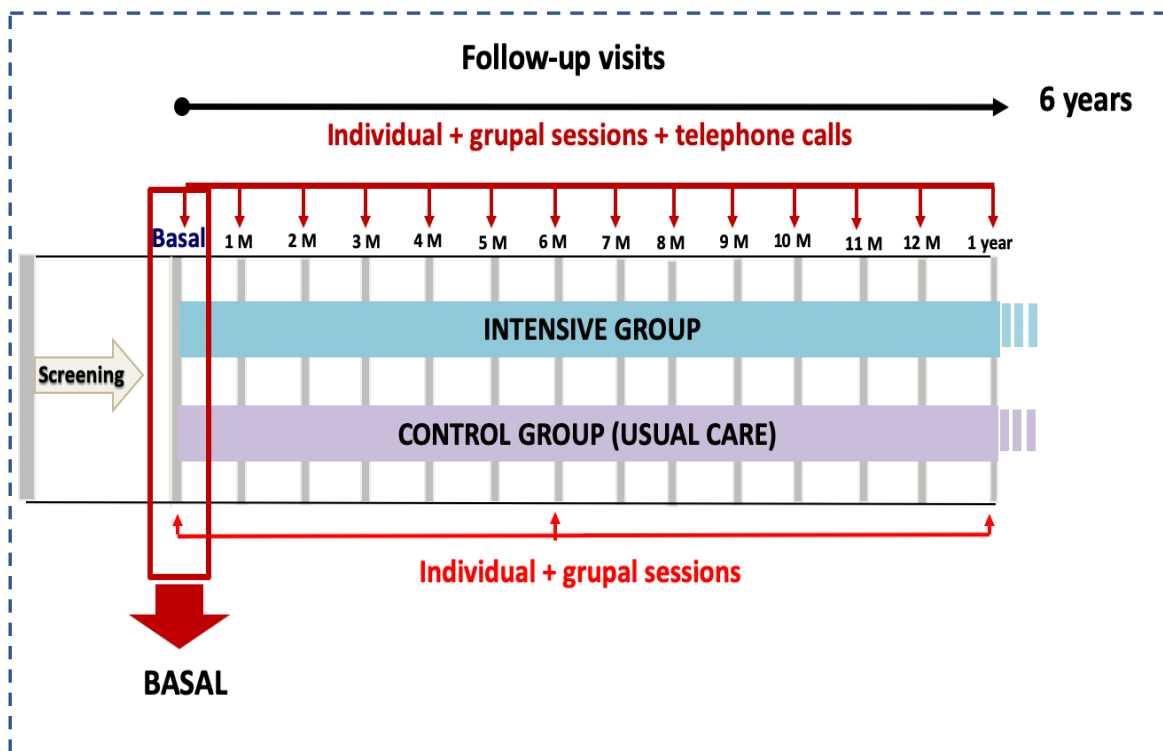
Figure 7. Study design from PREDIMED-Plus study.

2.   **Study design and population**

The study recruited a total of 6,874 individuals from 23 centres from Spain (Figure 8). The present study only included participants from one (Navarra-Nutrition) out of 23 centres of the project. Eligible participants were adults aged between 55-75 for men and 60-75 for women who were overweight or obese (BMI ≥27 and <40 kg/m^2) and at least presented three components of MetS considering the harmonized criteria [197]. Individuals were randomized in a 1:1 ratio to the intervention group or control group. The randomization considered the stratification by centre, sex and age in a block of 6 individuals, while for participants couples (living in the same household), the randomization was done by cluster [195,196]. Institutional Review Boards of each centre have approved the protocol and procedures (Ethic Committee for clinical investigations of the University of Navarra, 053/2013 and the Navarra Health Service 23/2013), following the ethical standards of the Declaration of Helsinki. All participants gave written informant consent. The experimental design used in this thesis is explained in the materials and methods of each article. Also, it is described according to Chapters in Figure 9.
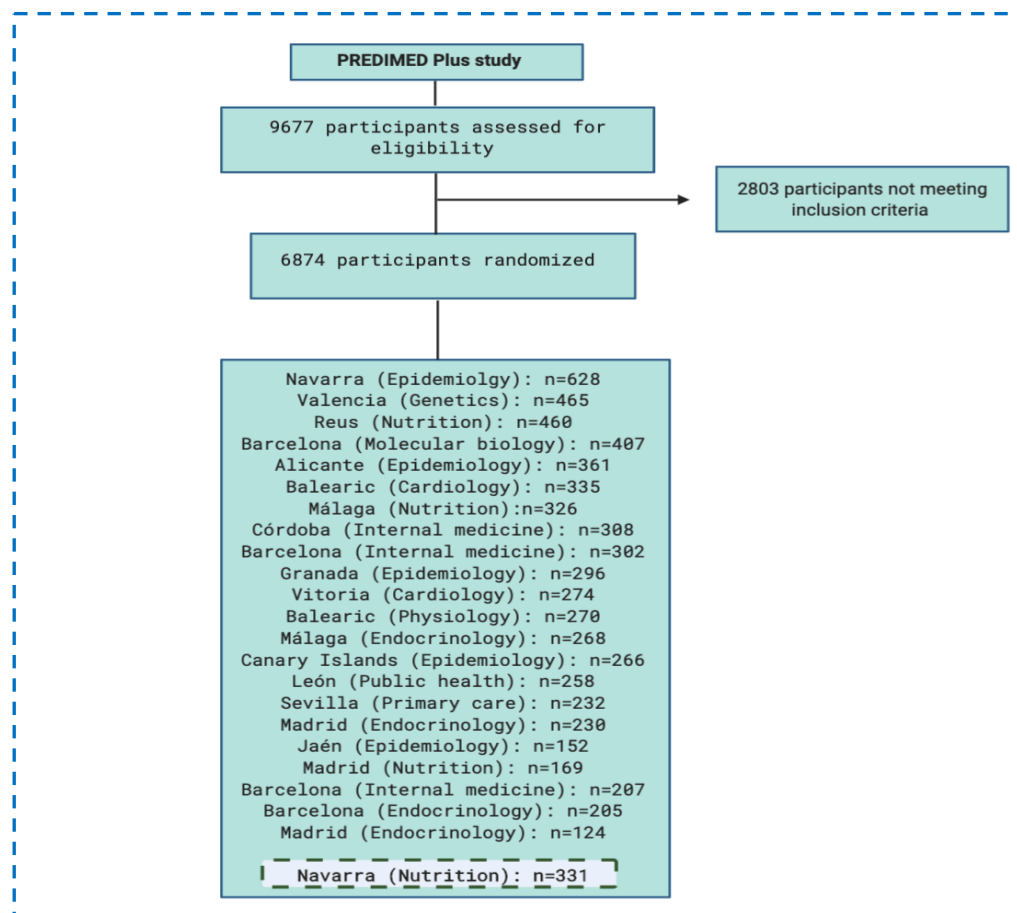
Figure 8. Flow chart of the PREDIMED-Plus study. Participants (6874) were randomized in 23 Spain centres. The present study includes 331 participants from Navarra-Nutrition centre.
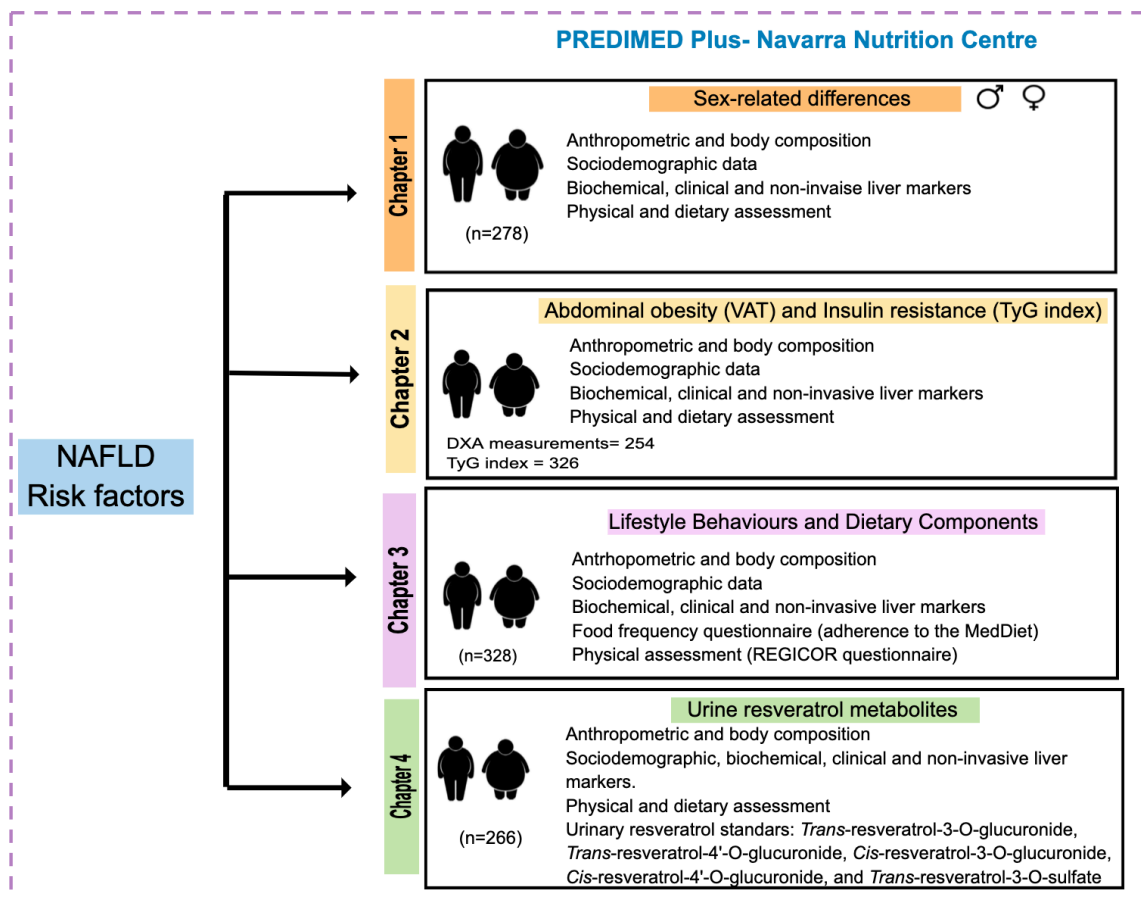
Figure 9. Study design for chapters 1 to 4 from PREDIMED-Plus study. Abbreviations: VAT, visceral adipose tissue; TyG, triglyceride and glucose index, MedDiet, Mediterranean diet; REGICOR, Registre Gironi del Cor questionnaire.

Table 7. Inclusion and exclusion recruitment criteria of participants in PREDIMED-Plus

Inclusion

Age: men (55-75 years); women (60-75 years)

BMI: ≥27 and <40 kg/m^2

Having at least three criteria for MetS:

- Increased waist circumference ≥102 for males and ≥88 for females
- Elevated triglycerides or treatment ≥ 150 mg/dL (1.7 mmol/L)
- Elevated fasting glucose or drug treatment ≥ 100 mg/dL
- Higher blood pressure or antihypertensive drug treatment, systolic ≥130 and/or diastolic ≥85 mm Hg
- Reduced HDL-c or drug treatment <40 mg/dL for men and <50 mg/dL in women

Exclusion

- Previous history of previous CVD
- Active cancer or history of malignancy within the last 5 years
- Inclusion and/or participation in another weight loss program in the six months before the selection visit.
- History of bowel resection and inflammatory bowel disease
- Obesity caused for endocrine origin (except hypothyroidism)
- Food allergy.
- Immunodeficiency or HIV-positive status.
- Cirrhosis or liver failure.
- Serious psychiatric disorders
- Alcohol abuse (or total daily alcohol intake >50 g) and drug addictions
- Concurrent therapy with immunosuppressive drugs or cytotoxic agents, corticosteroids and weight loss treatment.
- Individuals with an acute infection or inflammation.

3.   Data collection

3.1. Anthropometry, body composition and another covariate assessment

Trained nutritionists measured weight and height using anthropometric tape and calibrated stadiometer, respectively following the PREDIMED-Plus standardized protocol [195]. Waist circumference is measured midway between the lowest rib and the iliac crest and BMI was calculated from weight (kg) divided by height (meters) squared [195,196]. Body composition (total, trunk, android, gynoid and visceral fat mass) was performed by instructed personnel using a Dual-energy X-ray absorptiometry scan (DXA, Lunar iDXA, Madison, WI, USA; connected with enCore software, version 6) [198]. Sociodemographic, lifestyle, history of illness information was collected in a general study questionnaire by the nutritionist. Blood pressure was measured in triplicate using a validated semiautomatic oscillometer (Omron HEM 297 705C, Netherlands) [195] [196]. T2DM was established as the previous diagnosis of diabetes or HbA1C ≥6.5%, taking anti-diabetic treatment or fasting plasma glucose ≥126 mg/dL (7.0 mmol/L) described in the American Diabetes Association guidelines [199].

3.2. Biochemical and glucose homeostasis assessment

Nursing staff collected fasting blood after an overnight fast to determinate levels of total cholesterol, HDL-c, triglycerides, glucose, insulin, HbA1c, liver function parameters (alkaline phosphatase, ALT, AST, GGT, FGF-21), albumin, platelets and morning spot urine in situ. Blood and urine samples were stored in EDTA plasma, Citrate plasma, and serum aliquots and immediately frozen at -80°C [195]. Moreover, LDL-c and the VLDL-c were calculated by Friedewald formula [200]. Glucose markers such as the HOMA-IR [201], the homeostasis model assessment for β-cell function (HOMA-%B) [202], the fasting glucose-insulin ratio (FGIR) [203] and the fasting insulin resistance index (FIRI) [203] were determinate by the following formulas:

$$HOMA - IR = Insulin(mU/L) * glucose\ (mmol/L)/22.5$$

$$HOMA - \%B = \ Insulin(mU/L)/glucose\ (mmol/L) - 3.5$$

$$FGIR = glucose\ (mmol/L)/Insulin(mU/L)$$

$$FIRI = Insulin(mU/L) * glucose\ (mmol/L)\ /25$$

3.3. Physical activity assessment

Leisure-time physical activity (MET-min/week) was assessed using the validated short REGICOR (Registre Gironi del Cor) tool [195,204]. The REGICOR questionnaire includes information about type of activity, frequency (number of days) and duration (min/day) performed of the physical activity during the month [204]. This questionnaire evaluated the total energy expenditure considering the physical activity intensity in light (< 4 MET), moderate (4–5.5 MET), and vigorous (≥ 6 MET) [204,205].

3.4. Dietary assessment

Trained dietitians delivered a validated 143-item food frequency questionnaire during face-to-face visit [195,196]. This FFQ was validated in the Spanish population and used in the PREDIMED study [206]. Also, in the PREDIMED-Plus study, a 17-point scale questionnaire [195] was evaluated to assess the adherence to the energy-restricted MedDiet that is a modified version of the validated 14-point Mediterranean Diet Adherence Screener (MEDAS) [207,208] (Table 8). The 17-point scale questionnaire use more restrictive cut-offs for some caloric foods and includes some additional items to better acquirement the caloric reduction, which should be applied to a MedDiet adherence with the goal of weight loss. Moreover, for the present study, a modified version of a MedDiet adherence score constructed by Trichopoulou et al. was performed [209,210]. Healthy-beneficial food components include vegetables, fruits and mixed nuts, legumes, cereals, fish and seafood, and the ratio of monounsaturated fatty acid to a saturated fatty acid. For beneficial foods, individuals who had a consumption below the sex-specific median were assigned a value of 0, while participants whose consumption was above the median was assigned a value of 1. Also, food components presumed to be detrimental such as meat and dairy products, participants whose consumption was below the component sex-specific median were assigned 1 or 0 for consumption above the median. For the alcohol consumption, men who had an intake between 10 to <50 g/d and women consuming 5 to <25 g/d value of 1 was assigned and 0, otherwise. The MedDiet score ranged from 0 (minimum adherence) to 9 (maximal adherence). In this study, a score between 0-3 points was considered into low dietary adherence, between 4-5 points moderate adherence, and from 6 to 9 points high adherence.

Table 8. Energy- restricted MedDiet adherence 17-point scale

Questions	Criteria for 1 point
1. Do you use only extra-virgin olive oil for cooking, salad dressings, and spreads?	Yes
2. How many fruit units (including natural fruit juices) do you consume per day?	≥3
3. How many servings of vegetables/garden produce do you consume per day? [1 serving: 200 g (consider side dishes as half a serving)]	≥2 (≥1 portion raw or in a salad)
4. How many servings of white bread do you consume per day? (1 serving: 75 g)	≥1
5. How many times per week do you consume whole-grain cereals and pasta?	≥5
6. How many servings of red meat, hamburgers, or meat products do you consume per week? (1 serving: 100-150 g)	≥1
7. How many servings of butter, margarine or cream do you consume per week? (1 serving: 12 g)	<1
8. How many sugary beverages or sugar-sweetened fruit juices do you drink per week?	<1
9. How many servings of legumes do you consume per week? (1 serving: 150 g)	≥3
10. How many servings of fish or shellfish do you consume per week? (1 serving: 100-150 g of fish or 4-5 units or 200 g of shellfish)	≥3
11. How many times per week do you consume commercial sweets or pastries (not homemade)?	<3
12. How many servings of nuts (including peanuts) do you consume per week? (1 serving: 30 g)	≥3
13. Do you preferentially consume chicken, turkey or rabbit instead of beef, pork hamburgers or sausages?	Yes
14. How many times per week do you consume vegetables, pasta, rice or other dishes seasoned with sofrito (sauce made with tomato and onion, leek or garlic and simmered in olive oil)?	≥2
15. Do you avoid adding sugar to beverages (coffee, tea)?	Yes
16. How many times per week do you consume non-whole grain pasta or white rice?	<3
17. How many glasses of wine do you drink per day? (1 glass: 200 ml)	2-3 for male; 1-2 for female

Martínez-Gonzáles et al. [195]

3.5. Ultra-performance liquid chromatography coupled to triple quadrupole mass spectrometry (UPLC-QqQ-MS) analysis of urine resveratrol metabolites

Trans-resveratrol-3-O-glucuronide, *trans*-resveratrol-4'-O-glucuronide, *cis*-resveratrol-3-O-glucuronide, *cis*-resveratrol-4'-O-glucuronide and *trans*-resveratrol-3-O-sulfate standards were obtained from Toronto Research Chemicals (Toronto, ON, Canada). The resveratrol metabolites were extracted and quantified using a modified method previously described by Feliciano et al. [211]. The analytical method was validated according to the FDA guidelines (U.S. Department of Health and Human Services Food and Drug Administration guidance for industry bioanalytical methods validation). Briefly, 600μL of diluted urine samples (urine: water, 1:10) were thawed on ice and centrifuged at 15,000 x g for 15min at 4°C. Then the supernatant (350μL) was diluted with 4% phosphoric acid. The mixture (600μL) was loaded onto Oasis 96-well reversed-phase HLB (hydrophilic-lipophilic balanced) sorbent μ-SPE plates (Waters, Eschborn, Germany) and eluded with 60 μL of methanol after washing. Isotope labelled standards (±)-Catechin-2,3,4-13C3 (0.54mg/ml, Sigma-Aldrich, Steinheim, Germany) and Ferulic acid-1,2,3-13C3 (0.99mg/ml, Sigma-Aldrich, Steinheim, Germany) were spiked in samples before μ-SPE to indicate the recovery rate. Taxifolin (0.25mg/ml, Sigma-Aldrich, Steinheim, Germany) were used as internal standard. The identification and quantification of resveratrol metabolites was performed on a SHIMADZU Triple Quadrupole Mass Spectrometer (LCMS8060, SHIMADZU, Kyoto, Japan) through an electro-spray interface (ESI) source. Eluded samples (5μL) were injected through a Raptor Biphenyl column 2.1 x 50 mm, 1.8 μm (Restek, Bellefonte, USA) with a compatible Raptor Biphenyl Guard Cartridges 5 X 2.1mm (Restek, Bellefonte, USA) in the UPLC system). The mobile phases consisted of solvent A: water (HPLC grade, Sigma-Aldrich, Steinheim, Germany) with 0.1% formic acid (LC-MS grade, Thermo Fisher Scientific, Loughborough, UK), and solvent B: acetonitrile (HPLC grade, Sigma-Aldrich, Steinheim, Germany) with 0.1% formic acid. A fourteen minutes gradient joined by a two minutes equilibration was applied to the run under a flow rate of 0.5 mL/min at 30°C. The gradient was as follows (t(min), %B): (0,1), (1,1), (4,12), (8,12) (8.1,15), (11,15), (11.5,30), (12,99), (14,99), (14.1,1) (16,1). The MS/MS parameters and transitions of the target compounds were obtained in the optimization run. The resveratrol metabolites in samples were identified by comparing retention times with standards in corresponding Multiple Reaction Monitoring (MRM) transitions and quantified by calibration curves made from standard mixes. One pair of isomers *cis*-resveratrol-3-O-glucuronide and *cis*-resveratrol-4'-O-glucuronide were quantified together as they appear in the same retention time.

 The identification of each metabolite was based on the retention time of its corresponding pure standard following the same conditions and reference ion ratios based on the MS optimizations. Urinary resveratrol metabolites were normalized for urine creatinine concentrations (Figure 10).
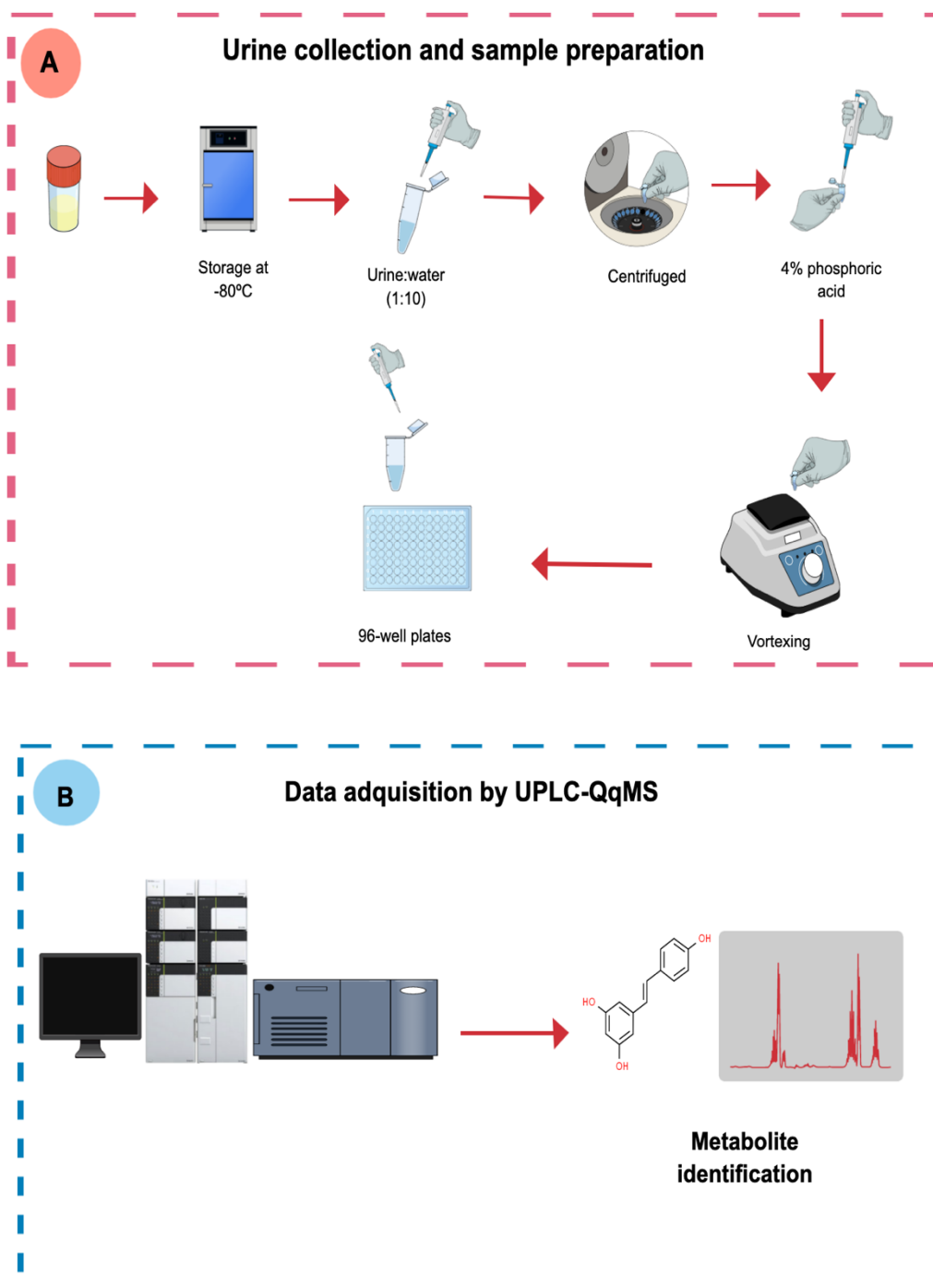
Figure 10. Experimental design describing the methodology process of urine resveratrol metabolite analysis (Chapter 5).

4.   Statistical analysis

Continuous variables are presented as means (standard deviation) and categorical variables as numbers (n) and percentages (%). Normality of variables was analyzed by Shapiro-Wilk and Kolmogorov-Smirnov test. To assess differences between categorical groups, Chi-square tests or Fisher's Exact test were used as appropriate. Moreover, correlations were evaluated using Pearson's coefficient for continuous variables or the Spearman's rank test for discrete variables. Differences between two groups were analyzed by Student's t-test or one-way analysis of variance (ANOVA) for more than two groups. For studying the relationship between cardiometabolic, liver parameters and general outcomes, we used multiple regression models in all Chapters. Also, we applied the ANCOVA test after adjustment for potential confounders to compare the adjusted means of groups with Bonferroni corrections for multiple comparisons.

To investigate interactions between sex and HSI, a 2 x 2 factorial analysis of the variables using the two-criteria ANOVA test was applied. Moreover, the area under the ROC curve (AUC) was performed to evaluate the predictive capacity of TyG index as a predictor of VAT considering as references values of 50th percentile of VAT specific by sex.

To explore the association between legume consumption and the HSI, it was performed multinomial logistic regression analyses adjusted for several confounders, and their effects were estimated using the relative risk ratio (RRR) with 95% confidence interval (CI).

Flexible cubic spline models were fit to evaluate the association between total urinary resveratrol metabolites (continuous variable) and liver enzymes (ALT, AST and GGT) levels above the upper limit normal. Models were adjusted by potential confounders. For total urinary resveratrol metabolites, we used 0 as a reference, with 4 and 3 knots.

The relatively big sample size used in the present analysis made it possible to comply with all the objectives set out in the present study, considering that type I and II errors can never entirely elude, but a larger sample size provides greater chances to detect the differences in the statistical tests. The statistical tests were two-tailed with a significance threshold of alpha=0.05. All statistical analyses were conducted with STATA version 12.0 or 16.0, StataCorp LP, College Station, TX, USA.

IV.   RESULTS

CHAPTER 1

Risk factors differentially associated with non-alcoholic fatty liver disease in males and females with metabolic syndrome

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CHAPTER 2

Relationship of visceral adipose tissue with insulin resistance surrogated and liver markers in individuals with metabolic syndrome chronic complications

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Relationship of visceral adipose tissue with surrogate insulin resistance and liver markers in individuals with metabolic syndrome chronic complications

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Abstract

Background: Visceral adipose tissue (VAT) has a hazardous influence on systemic inflammation, insulin resistance and an adverse metabolic profile, which increases the risk of developing non-alcoholic fatty liver disease (NAFLD) and chronic complications of diabetes. In our study we aimed to evaluate the association of VAT and the triglyceride glucose (TyG) as a proxy of insulin resistance surrogated with metabolic and liver risk factors among subjects diagnosed with metabolic syndrome (MetS).

Methods: A cross-sectional study was performed including 326 participants with MetS (55–75 years) from the PREDIMED-Plus study. Liver-status markers, VAT and TyG were assessed. Participants were stratified by tertiles according to VAT ($n = 254$) and TyG ($n = 326$). A receiver operating characteristic curve was used to analyse the efficiency of TyG for VAT.

Results: Subjects with greater visceral fat depots showed worse lipid profile, higher homeostatic model assessment for insulin resistance (HOMA-IR), TyG, alanine transaminase (ALT), fibroblast growth factor-21 (FGF-21), fatty liver index (FLI) and hepatic steatosis index (HSI) compared with participants in the first tertile. The multi-adjusted linear-regression analyses indicated that individuals in the third tertile of TyG ($>9.1–10.7$) had a positive association with HOMA-IR [$\beta = 3.07$ (95% confidence interval (CI) 2.28–3.86; p trend < 0.001)], ALT [$\beta = 7.43$ (95% CI 2.23–12.63; p trend $= 0.005$)], gamma glutamyl transferase (GGT) [$\beta = 14.12$ (95% CI 3.64–24.61; p trend $= 0.008$)], FGF-21 [$\beta = 190.69$ (95% CI 93.13–288.25; p trend < 0.001)], FLI [$\beta = 18.65$ (95% CI 14.97–22.23; p trend < 0.001)] and HSI [$\beta = 3.46$ (95% CI, 2.23–4.68; p trend < 0.001)] *versus* participants from the first tertile. Interestingly, the TyG showed the largest area under the receiver operating curve (AUC) for women (AUC $= 0.713$; 95% CI 0.62–0.79) compared with men (AUC $= 0.570$; 95% CI 0.48–0.66).

Conclusions: A disrupted VAT enlargement and impairment of TyG are strongly associated with liver status and cardiometabolic risk factors linked with NAFLD in individuals diagnosed with MetS. Moreover, the TyG could be used as a suitable and reliable marker estimator of VAT.

Keywords: insulin resistance, metabolic syndrome, non-alcoholic fatty liver disease, triglyceride glucose index, visceral adipose tissue

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Introduction

The metabolic syndrome (MetS) encompasses a cluster of cardiometabolic features like impaired glucose metabolism, dyslipidaemia, abdominal obesity, and elevated blood pressure.[1] The strong association between MetS and an increased risk of cardiovascular disease (CVD) as well as all-cause mortality is well documented.[2,3] On the other hand, non-alcoholic fatty liver disease (NAFLD) is recognized as the hepatic manifestation of MetS[4] that is related with insulin resistance and diabetes type 2 (T2DM).[5] NAFLD is a highly prevalent chronic liver illness, whose incidence linearly increases with body mass index (BMI) and adiposity.[6] This condition is quite common in obese individuals with central adiposity.[7,8] The distribution of adipose tissue is of great importance since abdominal obesity is a key factor in the development of the MetS[9] and NAFLD.[10] Insulin resistance is considered the primary triggering mechanism for the development of T2DM, NAFLD and MetS when fat accumulates in intra-abdominal depots[9,10] Thus, body-fat distribution in older adults is critical for determining how susceptible they are or will be to developing NAFLD and/or other CVD[11–14] being partly attributed to sex differences in fat content.[12,14] Central obesity is often quantified using waist circumference. But, it can be confounded by varying levels of subcutaneous fat in the waist, and may not accurately reflect visceral fat in all individuals.[15] The dual-energy X-ray absorptiometry (DXA) is a practical and valuable tool to assess visceral fat mass.[16] Nevertheless, the DXA equipment is expensive and might not be easy to access. In this sense, the identification of non-invasive markers able to discriminate subjects with higher visceral adiposity and higher susceptibility for developing NAFLD would be of great interest, as well as relating to T2DM complications. Indeed, liver biopsy is the gold standard for NAFLD diagnosis,[7] but it is an invasive technique not suitable for routine screening and monitoring.[7] Several non-invasive markers related to liver status and insulin resistance have been proposed in characterizing NAFLD.[7,17–19] A novel potential marker is triglyceride glucose (TyG), which has demonstrated a better predictive value compared with fasting plasma glucose (FPG) for the risk of T2DM in normoglycaemic individuals, as well as being associated with insulin resistance.[20] In the present study, the hypothesis was that subjects with a larger amount of VAT and increased TyG levels have higher susceptibility for showing adverse manifestations related to T2DM and development of NAFLD. Therefore, our primary objective was to assess the potential association of TyG with VAT, cardiometabolic risk factors, serum and NAFLD markers in overweight/obese individuals with MetS.

Materials and methods

Study population and design

This research is a cross-sectional study concerning baseline data from participants of the Navarra-Nutrition Centre within the PREDIMED-Plus trial (ISRCTN89898870; http://www.isrctn.com/ISRCTN89898870). PREDIMED-Plus is a multicentre, parallel-group, randomized trial carried out in Spain, aiming to evaluate the effectiveness of an energy-restricted traditional Mediterranean diet, physical activity promotion and behavioural support (intervention group) on the primary prevention of CVD, in comparison with general advised energy-unrestricted Mediterranean diet (control group). Detailed methods and protocols of the study have been published previously.[21,22] In brief, 6874 individuals were recruited in 23 Spanish centres. Eligible participants were men (55–75 years) and women (60–75 years) with a BMI $\geqslant 27 \, kg/m^2$ and $< 40 \, kg/m^2$ and fulfilling at least three criteria for the MetS: waist circumference (WC) in White people $\geqslant 102 \, cm$ for men and $\geqslant 88 \, cm$ for women, elevated triglycerides levels $\geqslant 150 \, mg/dl$ or drug treatment for hyperlipidemia; reduced high-density lipoprotein cholesterol (HDL-c) $< 40 \, mg/dl$ in men and $< 50 \, mg/dl$ in women or drug treatment; elevated blood pressure systolic $\geqslant 130 \, mmHg$ and/or diastolic $\geqslant 85 \, mmHg$ or current use of antihypertensive medication; elevated fasting glucose $\geqslant 100 \, mg/dl$ or drug treatment, according to guidelines from the International Diabetes Federation/National Heart, Lung and Blood Institute/American Heart Association (2009).[23] As described elsewhere, exclusion criteria included a background of alcohol overuse, liver injury, history of previous CVD, gastrointestinal or other disorders, infectious processes, therapy with immunosuppressive drugs, cytotoxic agents or systemic corticosteroids. The protocol and procedures were approved by the Research Ethic Committee for clinical investigations of the University of Navarra (053/2013) according to the Declaration of Helsinki. All participants provided written informed consent. At Navarra-Nutrition Centre, 331 were included in

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the study, of which 326 participants had available data to calculate TyG, and 254 patients were assessed by DXA.

Study assessment

Clinical and biochemical measurements

At baseline, participants completed an administered survey, which included questions about socio-demographic characteristics, lifestyle behaviours, disease history and medication. Smoking habits were classified into 'never', 'former' or 'current smoker', as described elsewhere.[21] Blood pressure was measured in triplicate using a validated semiautomatic oscillometer (Omron HEM-705CP, Netherlands). T2DM was established as previous diagnosis of diabetes or glycated haemoglobin (HbA1c) ≥6.5%, use of antidiabetic medication or fasting glucose ≥126 mg/dl according to the American Diabetes Association guidelines.[24] After overnight fasting for at least 12 h, a blood sample was obtained from each participant. Serum and plasma were collected and frozen at −80°C. All biochemical measurements, including plasma glucose, HbA1c, insulin, total cholesterol, HDL-c, triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) were performed using standard laboratory enzymatic methods and following validated protocols.[21] The fibroblast growth factor 21 (FGF-21) plasma concentrations were measured using human FGF-21 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) with an autoanalyzer system (Triturus, Grifols SA, Barcelona, Spain) following the manufacturer's instructions. Low-density lipoprotein cholesterol (LDL-c) concentration was calculated by Friedewald's formula and the very-low-density lipoprotein cholesterol (VLDL-c) was calculated as triglycerides / 5.[25] Also, homeostatic model assessment for insulin resistance (HOMA-IR) was calculated according to the formula: fasting insulin (mIU/l) × fasting glucose (nmol/l)/22.5.[26]

Dietary variables

Trained dietitians face-to-face administered a semi-quantitative 143-item food-frequency questionnaire to estimate energy intake and alcohol consumption.[27] Also, a 17-item questionnaire was implemented, which is a modified version of the previously validated questionnaire used in the PREDIMED study to assess the participant's adherence to the Mediterranean diet.[28]

Physical activity measurement

Physical activity was assessed using the short Registre Gironi del Cor questionnaire that showed high reliability and sensitivity in detecting changes in moderate and vigorous intensity.[29,30] This tool was validated in the Spanish adult population, which is a version of the Minnesota Leisure Time.[29] This questionnaire evaluated the total energy expenditure in leisure-time physical activity using Metabolic Equivalent Tasks (METs) in minutes/week. Physical activities were classified into light-intensity (<4 METs), moderate intensity (4.0–5.5 METs), and vigorous intensity (≥6 METs) as detailed in the report.[29] Sedentary lifestyles were evaluated using a validated Nurses' Health Study questionnaire.[31] For the present study, physical activity was expressed as MET hours/week.

Anthropometry and body composition measurements

Anthropometric measurements were performed by trained dietitians following standardized PREDIMED-Plus protocols.[21] Weight, height and waist circumference (WC), were measured using a calibrated scale, a stadiometer and an anthropometric tape, respectively. BMI was conventionally calculated as weight in kilograms divided by the height in square metres (kg/m^2). VAT was estimated using dual-energy X-ray absorptiometry (Lunar iDXA™, software version 6.0, Madison, WI, USA) connected with enCore™ software, which was assessed by trained operators according to standard procedures supplied by the manufacturer.

Non-invasive markers

TyG is a newly described marker reported as a useful screening tool for surrogated insulin resistance,[20,32,33] NAFLD,[34] and as an early predictor of MetS features.[35] This marker was calculated using biochemical data according to the following formula (Equation 1):[32,36]

$$TyG = Ln[triglyceride\,(mg\,/\,dl)* \; glucose\,(mg\,/\,dl)\,/\,2] \qquad \text{Equation 1.}$$

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The hepatic steatosis index (HSI; Equation 2) was validated in a cohort of patients with NAFLD diagnosed by ultrasonography.[37]

$$HSI = 8 \times ALT / AST \text{ ratio} + BMI(+2, \text{ if diabetes}; +2, \text{ if female})^{[37,38]}$$

Equation 2.

HSI was also computed to estimate liver status. Another liver marker as an indicator of NAFLD is the fatty liver index (FLI), which was calculated as previously described (Equation 3)[39] by:

$$\frac{e^{0.953\times\log(\text{triglycerides})+0.139\times BMI+0.718\times\log(GGT)+0.053\times\text{waist circumference}-15.745}}{1+e^{0.953\times\log(\text{triglycerides})+0.139\times BMI+0.718\times\log(GGT)+0.053\times\text{waist circumference}-15.745}} \times 100$$

Equation 3.

Statistical analyses

We retrospectively estimated the sample size to find differences between groups with a precision of 0.40 and a standard deviation (SD) of 0.5, and $\alpha = 0.05$. The statistical power of the study was 90%. Continuous variables are presented as means $\pm$ SD and categorical variables as numbers (n) and percentages (%). One-way analysis of variance and Chi-square tests or Fisher's exact test for categorical variables were used to assess differences between groups, as appropriate. The analysis of covariance test after adjustment was used for the following potential confounders: age (years), physical activity (MET hours/week), energy intake (kcal/d), alcohol intake (g) and smoking status (never, former, current). Bonferroni correction was applied to assess differences in metabolic and liver parameters according to sex-specific VAT tertiles. VAT for men: T1 (1.29 to ≤2.42), T2 (>2.42 to ≤3.10), T3 (>3.10 to 5.45); VAT for women: T1 (0.77 to ≤1.60), T2 (>1.60 to ≤2.06), T3 (>2.06 to 3.59). Crude and multiple linear regression models adjusted by age (years), sex (male and female), physical activity (MET hours/week), energy intake (kcal/d), alcohol intake (g) and smoking status (never, former, current) were fitted to statistically analyse the association between NAFLD biomarkers and tertiles of TyG. Tests of linear trend were assessed assigning the median value of each tertile of TyG and then using it as a continuous variable and correlation was assessed using the Pearson's coefficient. The area under the receiver operating characteristics (ROC) curve

(AUC) was performed to quantify the value of TyG as a predictor of VAT, considering as reference values the 50th percentile of VAT by sex. All tests were two sided, and cut-off level of significance was defined as 0.05. Statistical analyses were carried out with Stata 12.0 software (StataCorp LP, College Station, TX, USA).

Results

Study sample characteristics

Baseline characteristics of men and women according to VAT sex-specific tertiles are summarized in Table 1. As expected, BMI and WC increased across VAT tertiles. No significant differences were found in the frequency of diabetes, hypertension and smoking habits among tertiles in both sexes. Likewise, blood pressure [systolic blood pressure (SBP) and diastolic blood pressure (DBP)] measurements, energy intake, alcohol consumption, adherence to the Mediterranean diet score and physical activity did not differ statistically.

Crosstalk between VAT, TyG and NAFLD risk factors

Anthropometric, metabolic profile and liver status of participants are reported in Table 2. The adjusted analysis revealed that BMI and WC were significantly increased through VAT tertiles specific by sex. Moreover, insulin, TyG and HOMA-IR increased with VAT tertiles reaching statistical differences among them (all $p < 0.05$). Glucose and HbA1c did not show differences between tertiles. As concerns lipid markers, the T3 group presented significantly higher levels of VLDL-c [mean 32.4 mg/dl (95% CI 29.7−35.2)], triglycerides [mean 162.1 mg/dl (95% CI 148.3−175.9)] and triglyceride (TG)/HDL-c ratio [mean 3.8 mg/dl (95% CI 3.4−4.3)] than T1 participants, while no associations were found regarding total cholesterol, LDL-c and HDL-c serum levels. Participants in the highest VAT tertile showed significantly higher ALT levels, HSI and FLI scores as compared with subjects in the lowest tertile of VAT. No significant differences were found in AST and FGF-21 levels in VAT tertiles.

The association of TyG with variables related to liver health was explored (Table 3). Linear regression models were fitted considering NAFLD-related markers as dependent factors and TyG as

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Table 1. Clinical and lifestyle characteristics of subjects with MetS according to tertiles of VAT by sex.

| | Tertiles of visceral adipose tissue (kg) | | | | | | | | |
| | Men (n=133) | | | | Women (n=121) | | | |
	T1 (n=45) (1.29 to ≤2.42)	T2 (n=44) (>2.42 to ≤3.10)	T3 (n=44) (>3.10 to 5.45)	p value*	T1 (n=41) (0.77 to ≤1.60)	T2 (n=40) (>1.60 to ≤2.06)	T3 (n=40) (>2.06 to 3.59)	p value*
Age (years)	63.9 ± 5.8	64.5 ± 5.8	64.6 ± 5.1	0.818	67.7 ± 3.5	67.0 ± 4.2	67.5 ± 4.4	0.751
BMI (kg/m^2)	30.0 ± 2.2	31.5 ± 2.2	34.0 ± 2.9	<0.001	30.6 ± 2.9	32.4 ± 3.6	34.4 ± 3.2	<0.001
WC (cm)	103.5 ± 5.8	109.5 ± 6.2	117.3 ± 7.6	<0.001	97.1 ± 6.5	102.8 ± 6.9	109.5 ± 7.2	<0.001
VAT (kg)	2.0 ± 0.3	2.8 ± 0.2	3.8 ± 0.5	<0.001	1.3 ± 0.2	1.8 ± 0.1	2.5 ± 0.4	<0.001
Diabetes, n (%)	19 (42.2)	16 (36.4)	17 (38.6)	0.849	10 (24.4)	16 (40.0)	19 (47.5)	0.089
Hypertension, n (%)	40 (88.9)	42 (95.5)	42 (95.5)	0.362	39 (95.1)	38 (95.0)	37 (92.5)	0.851
SBP (mmHg)	147.6 ± 18.5	142.3 ± 13.8	143.4 ± 14.7	0.250	145.8 ± 16.9	143.5 ± 15.8	142.0 ± 16.2	0.578
DBP (mmHg)	88.5 ± 9.9	88.6 ± 8.5	88.0 ± 6.7	0.941	85.1 ± 8.9	87.6 ± 9.9	86.7 ± 8.6	0.455
Smoking habits, n (%)				0.092				0.677
Never smoker	12 (26.7)	7 (15.9)	4 (9.1)		30 (73.2)	23 (57.5)	25 (62.5)	
Former smoker	23 (51.1)	26 (59.1)	34 (77.3)		8 (19.5)	13 (32.5)	11 (27.5)	
Current smoker	10 (22.2)	11 (25.0)	6 (13.6)		3 (7.3)	4 (10.0)	4 (10.0)	
Alcohol intake (g/d)	15.2 ± 14.3	18.5 ± 20.3	23.3 ± 20.6	0.119	2.4 ± 6.2	5.3 ± 8.3	2.6 ± 4.9	0.102
Energy intake (kcal/d)	2655.4 ± 580.2	2670.0 ± 439.7	2789.0 ± 550.2	0.428	2451.5 ± 549.5	2407.0 ± 511.9	2474.2 ± 413.4	0.827
Adherence to MedDiet (0–17 points)	9 ± 2.7	8.5 ± 2.2	8.8 ± 2.5	0.636	9.2 ± 2.5	9.0 ± 2.8	9.4 ± 2.5	0.767
Physical activity (MET hours/week)	59.0 ± 57.6	61.2 ± 49.6	46.9 ± 41.1	0.356	43.2 ± 33.6	39.4 ± 32.9	36.0 ± 26.5	0.588

*p value for differences between tertiles of visceral fat mass by sex was calculated by Chi-square, Fisher's exact test or ANOVA, as appropriate.
p<0.05 is considered statistically significant. Data are expressed as mean ± SD.
ANOVA, analysis of variance; BMI, body mass index; DBP, diastolic blood pressure; MedDiet, Mediterranean diet; MET, metabolic equivalent; MetS, metabolic syndrome; SBP, systolic blood pressure; SD, standard deviation; WC, waist circumference; VAT, visceral adipose tissue.

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Table 2. Anthropometric, body composition, metabolic profile and liver status in subjects with MetS according to VAT sex-specific tertiles.

	Tertiles of visceral adipose tissue (kg)			*p* value
	T1	T2	T3	
	(*n* = 86)	(*n* = 84)	(*n* = 84)	
Men	(1.29 to ≤2.42)	(>2.42 to ≤3.10)	(>3.10 to 5.45)	
Women	(0.77 to ≤1.60)	(>1.60 to ≤2.06)	(>2.06 to 3.59)	
Total	(0.77 to 2.42)	(>1.60 to 3.10)	(>2.06 to 5.45)	
Anthropometric and body composition				
BMI (kg/m²)	30.3 (29.7–30.9)[a,b,c]	32.0 (31.4–32.6)[b,c]	34.1 (33.5–34.8)	<0.001
WC (cm)	101.0 (99.5–102.6)[a,b,c]	106.3 (104.8–107.8)[b,c]	113.1 (111.5–114.6)	<0.001
VAT (kg)	1.7 (1.6–1.8)[a,b,c]	2.3 (2.2–2.4)[b,c]	3.1 (3.0–3.2)	<0.001
Glucose profile				
Glucose (mg/dl)	115.1 (108.0–122.2)	118.2 (111.2–125.2)	123.6 (1116.6–130.6)	0.247
HbA1c (%)	6.0 (5.8–6.2)	6.2 (6.0–6.4)	6.2 (6.0–6.5)	0.281
TyG	8.8 (8.7–8.9)[a,b,c]	9.0 (8.9–9.1)	9.1 (9.0–9.2)	0.001
Insulin (mU/l)	10.2 (8.5–11.9)[a,b,c]	13.8 (12.1–15.4)	16.5 (14.9–18.2)	<0.001
HOMA-IR	2.9 (2.3–3.4)[a,b,c]	4.1 (3.5–4.6)	5.0 (4.5–5.5)	<0.001
Lipid profile				
Total cholesterol (mg/dl)	198.0 (190.2–205.8)	201.2 (193.4–209.0)	205.6 (197.7–213.5)	0.411
LDL-c (mg/dl)	125.5 (118.5–132.5)	125.8 (118.7–132.9)	129.1 (121.8–136.4)	0.747
HDL-c (mg/dl)	47.8 (45.6–49.9)	45.5 (43.3–47.6)	45.9 (43.7–48.1)	0.315
VLDL-c (mg/dl)	25.0 (22.2–27.7)[a,b,c]	30.2 (27.4–32.9)	32.4 (29.7–35.2)	<0.001
Triglycerides (mg/dl)	124.8 (111.0–138.5)[a,b,c]	150.9 (137.2–164.5)	162.1 (148.3–175.9)	<0.001
TG/HDL-c ratio	2.9 (2.4–3.3)[a,c]	3.6 (3.1–4.0)	3.8 (3.4–4.3)	0.008
Liver status				
ALT (U/l)	23.3 (19.1–27.4)[a,c]	29.2 (25.1–33.3)	32.0 (27.9–36.1)	0.013
AST (U/l)	21.8 (19.0–24.6)	24.2 (21.4–26.9)	25.0 (22.2–27.7)	0.268
GGT (U/l)	37.2 (28.7–45.6)	46.8 (38.5–55.2)	40.7 (32.2–49.2)	0.272
FGF-21 (pg/ml)*	378.5 (294.5–462.5)	484.7 (403.8–565.6)	430.5 (346.7–514.4)	0.207
FLI (arbitrary units)	66.6 (63.9–69.3)[a,b,c]	79.8 (77.1–82.5)[b,c]	86.9 (84.2–89.6)	<0.001
HSI (arbitrary units)	40.2 (39.4–41.1)[a,b,c]	43.2 (42.2–44.1)[b,c]	45.9 (45.1–46.8)	<0.001

*FGF-21 available in 211 patients.
p < 0.05 is considered statistically significant. Data are expressed as mean (95% CI). Variables were adjusted by age (years), physical activity (MET hours/week), energy intake (kcal/d), alcohol intake (g) and smoking status (never, former, current).
Data is stratified by VAT sex- specific tertiles.
[a,b]Significant differences between T1 vs T2.
[a,c]Significant differences between T1 vs T3.
[b,c]Significant differences between T2 vs T3.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FGF-21, fibroblast growth factor- 21; FLI, fatty liver index; GGT, gamma-glutamyl transferase; HbA1c, glycated haemoglobin A1c; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; HSI, hepatic steatosis index; LDL-c, low-density lipoprotein cholesterol; MET, Metabolic Equivalent Task; MetS, metabolic syndrome; TG/HDL ratio, triglycerides/high-density lipoprotein ratio; TyG, triglyceride glucose; VAT, visceral adipose tissue; VLDL-c, very-low-density lipoprotein cholesterol; WC, waist circumference.

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Table 3. Multivariable linear regression analyses evaluating the association between TyG tertiles as independent variable and liver status as dependent variable.

	Tertiles of TyG			*p* for trend
	T1	T2	T3	
	(*n* = 109)	(*n* = 110)	(*n* = 107)	
	(7.3–8.7)	(>8.7–9.1)	(>9.1–10.7)	
	β estimates (95% CI)	β estimates (95% CI)	β estimates (95% CI)	
WC (cm)				
Crude	(0 Ref.)	1.32 (–1.06 to 3.71)	3.21 (0.81–5.61)	0.009
Multivariable adjusted	(0 Ref.)	1.81 (–0.37 to 3.99)	2.62 (0.41–4.84)	0.020
HOMA-IR				
Crude	(0 Ref.)	1.21 (0.43–1.99)	3.09 (2.31–3.88)	<0.001
Multivariable adjusted	(0 Ref.)	1.25 (0.48–2.01)	3.07 (2.28–3.86)	<0.001
ALT (U/l)				
Crude	(0 Ref.)	3.79 (–1.48 to 9.07)	8.14 (2.83–13.45)	0.003
Multivariable adjusted	(0 Ref.)	4.79 (–0.32 to 9.90)	7.43 (2.23–12.63)	0.005
AST (U/l)				
Crude	(0 Ref.)	1.84 (–1.51 to 5.18)	2.56 (–0.80 to 5.93)	0.137
Multivariable adjusted	(0 Ref.)	2.29 (–1.00 to 5.57)	1.98 (–1.36 to 5.33)	0.246
GGT (U/l)				
Crude	(0 Ref.)	3.06 (–7.39 to 13.51)	16.72 (6.17–27.26)	0.002
Multivariable adjusted	(0 Ref.)	4.07 (–6.21 to 14.34)	14.12 (3.64–24.61)	0.008
FGF-21 (pg/ml)*				
Crude	(0 Ref.)	92.45 (–2.17 to 187.07)	195.11 (100.49–289.73)	<0.001
Multivariable adjusted	(0 Ref.)	91.26 (–5.09 to 187.62)	190.69 (93.13–288.25)	<0.001
FLI (arbitrary units)				
Crude	(0 Ref.)	11.18 (7.46–14.90)	19.60 (15.84–23.36)	<0.001
Multivariable adjusted	(0 Ref.)	11.78 (8.18–15.39)	18.65 (14.97–22.33)	<0.001
HSI (arbitrary units)**				
Crude	(0 Ref.)	1.76 (0.52–3.00)	3.35 (2.11–4.60)	<0.001
Multivariable adjusted	(0 Ref.)	1.95 (0.75–3.16)	3.46 (2.23–4.68)	<0.001

*FGF-21 available in 278 patients.
**Adjusted for all variables except for sex.
p < 0.05 was considered statistically significant. Data are expressed as mean (95% CI). Models were adjusted by age (years), sex (male and female), physical activity (MET hours/week), energy intake (kcal/d), alcohol intake (g) and smoking status (never, former, current).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; FGF-21, fibroblast growth factor-21; FLI, fatty liver index; GGT, gamma-glutamyl transferase; HOMA-IR, homeostatic model assessment for insulin resistance; HSI, hepatic steatosis index; MET, Metabolic Equivalent Task; Ref., reference; TyG, triglyceride glucose; WC, waist circumference.

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the independent variable (Table 3). A fully adjusted model revealed that individuals in the third TyG tertile ($>9.1-10.7$) were significantly associated with higher WC ($\beta = 2.62$; 95% CI 0.41–4.84, p for trend $= 0.020$), HOMA-IR ($\beta = 3.07$; 95% CI 2.28–3.86, p for trend < 0.001), ALT ($\beta = 7.43$; 95% CI 2.23–12.63, p for trend $= 0.005$), GGT ($\beta = 14.12$; 95% CI 3.64–24.61, p for trend $= 0.008$), FGF-21 levels ($\beta = 190.69$; 95% CI 93.13–288.25, p for trend < 0.001), FLI units ($\beta = 18.65$; 95% CI 14.97–22.33, p for trend < 0.001), HSI units ($\beta = 3.46$; 95% CI 2.23–4.68, p for trend < 0.001) than participants in the first TyG tertile. Furthermore, variables associated with glucose–insulin homeostasis were significantly correlated with VAT except for men in glucose levels (Figure 1). Glucose (men: $r = 0.093$, $p = 0.292$; women: $r = 0.264$, $p = 0.004$) [Figure 1(a)], triglycerides (men: $r = 0.291$, $p < 0.001$; women: $r = 0.220$, $p = 0.016$) [Figure 1(b)], HOMA-IR (men: $r = 0.345$, $p < 0.001$; women: $r = 0.500$, $p < 0.001$) [Figure 1(c)], and TyG (men: $r = 0.266$, $p = 0.002$; women: $r = 0.322$, $p < 0.001$) [Figure 1(d)].

Receiver operating characteristic (ROC) analyses for TyG to predict VAT

ROC curves were applied to assess the capacity of TyG to identify elevated VAT accumulation in both sexes (Figure 2). The AUCs of the TyG for prediction of VAT was 0.570 (95% CI 0.48–0.66) for men and 0.713 (95% CI 0.62–0.79) for women.

Discussion

In this translational study, VAT and TyG were associated with relevant liver and cardiometabolic risk factors linked to NAFLD and insulin resistance in subjects with MetS. Moreover, TyG could be a reliable indicator of visceral fat mass. Many metabolic abnormalities related to insulin resistance often occur in obese individuals with higher amount of VAT.[9,40] The link between altered VAT triggering with a disorder in glucose and insulin metabolism may appear to be a driving factor in T2DM and NAFLD.[4,5]

Interestingly, TyG and atherogenic lipid profiles (VLDL-c, triglycerides and TG/HDL-c ratio) were significantly increased across tertiles of sex-specific VAT independently of confounding

factors. In line with our results, Lee and colleagues observed VAT and triglycerides being independent risk factors for hepatic steatosis.[41] VAT is the main source of free fatty acids (FFAs) and other biological compounds, which enter the portal circulation and contribute to hepatic fat accumulation,[40] insulin resistance[4,5] and glucose intolerance, promoting a decreased hepatic insulin sensitivity, increasing the risk of developing T2DM and NAFLD.[4] Moreover, a statically significant increase of non-invasive hepatic markers (ALT, FLI and HSI) in participants with higher fat-storage capacity in VAT was found. Previously, studies demonstrated that increased VAT was associated with higher ALT levels[42] or significant fibrosis in subjects diagnosed with NAFLD.[43] Based on these data, central adiposity plays a key role in NAFLD pathogenesis[10,14,44] promoting liver damage,[43] insulin resistance and disrupted lipid metabolism.[45]

Currently, NAFLD has become a public health problem with a negative impact over the individual's health, socioeconomic and healthcare system.[6,46] In this context, early screening is crucial in the NAFLD pathogenesis,[7] as it is an overlooked T2DM complication.[5] Liver biopsy is the gold standard for NAFLD diagnosis.[7] However, it has several limitations, such as sampling error, cost, medical complications and technical difficulties.[47] In this regard, several methodologies have been used in the detection and featuring of NAFLD shown to be relatively effective, inexpensive and useful in a primary healthcare setting.[19,47–49] TyG is a novel marker exhibiting accuracy for recognizing insulin resistance and diabetes-related manifestations.[20,50] Furthermore, this marker was found highly sensitive for detecting NAFLD.[34] Simental-Mendía *et al.* suggested that the best TyG level for diagnosis of insulin resistance was Ln 4.65, which showed the highest sensitivity (84.0%) and specificity (45.0%) values.[36] Interestingly, the multivariable regression analysis demonstrated that individuals with a higher TyG (>9.1) value were associated with higher levels of HOMA-IR ALT, GGT, FGF-21, FLI and HSI units compared with lower TyG values ($\leqslant 8.7$), after adjusting for potential confounders, which confirms the relationship with inflammation and T2DM. Evidence supports T2DM is an important risk factor for NAFLD,[5,6] which is characterized by a resistance of insulin action in targets tissues and a disruption of the beta cells in the pancreatic islets to secrete enough insulin to

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Men   Women
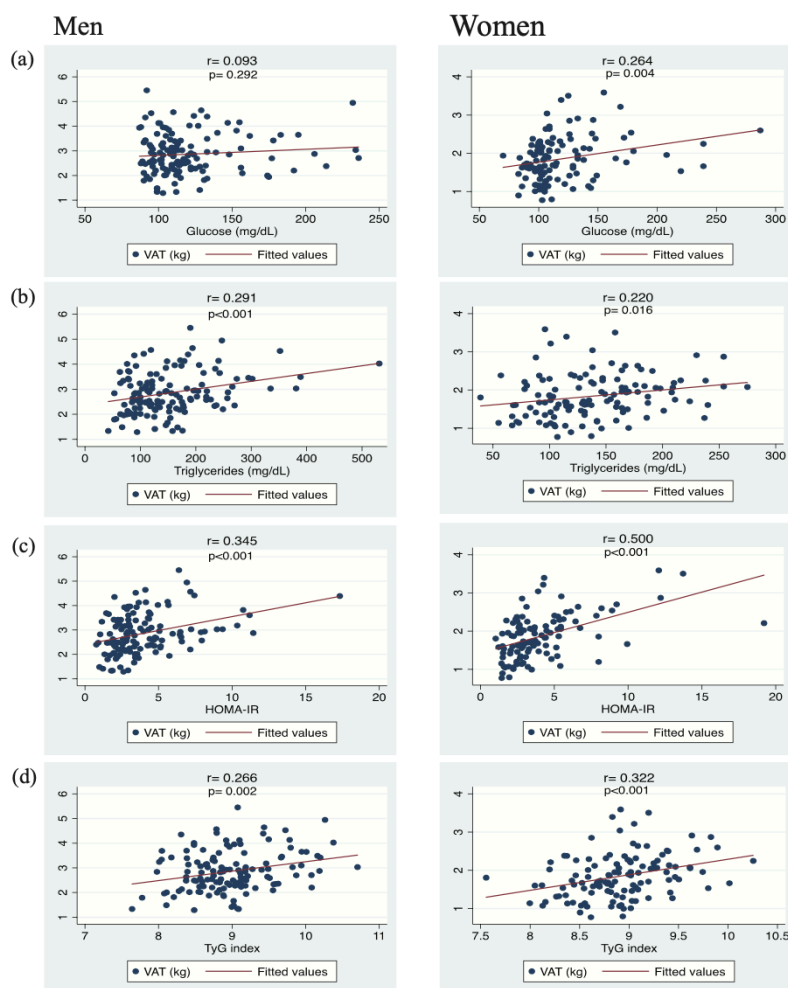
Figure 1. Correlations between VAT and parameters related to glucose and insulin homeostasis in subjects with MetS according to sex.
MetS, metabolic syndrome; VAT, visceral adipose tissue.

overcome this resistance.[24] The prevalence of NAFLD and non-alcoholic steatohepatitis (NASH) in individuals diagnosed with T2DM equates to over 60% increased risk of NAFLD pathogenesis and mortality.[6] These results suggested that insulin resistance is an important contributor to the development of NAFLD.[4] This finding is similar to results from Bonnet *et al.*, who reported that increased levels of ALT and GGT are strongly associated with hepatic insulin resistance and decreased hepatic insulin clearance.[51] Another liver marker is the FGF-21, primarily produced in

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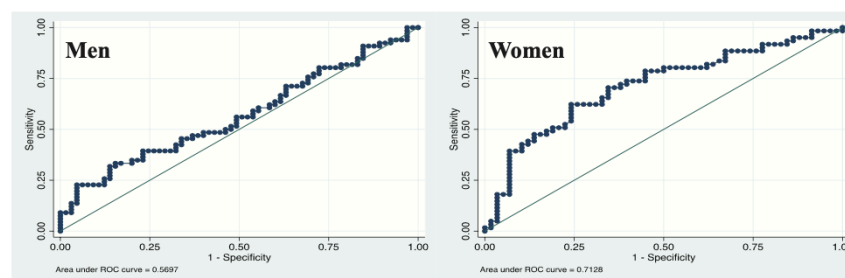
Figure 2. Receiver operating characteristics (ROC) curve analysis of predictive value of the TyG in subjects with MetS according to sex.
VAT cut-off men: ≥2.777 kg; VAT cut-off women: ≥1.748 kg.
MetS, metabolic syndrome; TyG, triglyceride glucose; VAT, visceral adipose tissue.

hepatocytes and implicated in the regulation of glucose–lipid metabolism, insulin sensitivity, inflammation and energy homeostasis.[52] Several clinical studies and reviews have documented that disrupted adipose tissue and excessive intrahepatic fat accumulation may trigger FGF-21 resistance.[52,53] Thus, Shen and colleagues[54] found that NAFLD patients showed significantly higher serum FGF-21 levels compared with subjects without NAFLD.[54] Furthermore, the present study showed that subjects with higher values of TyG had 3.46 more units of HSI compared with reference (lower values). Taken together, these results can be explained by insulin resistance being a major feature of NAFLD that works by increasing *de novo* lipogenesis and FFA flux to the liver through decreased inhibition of lipolysis,[4] promoting inflammation, oxidative stress[55] and hepatocyte injury.[56] Thus, individuals with pre-diabetes and T2DM represent an at-high-risk population where early diagnosis of NAFLD is crucial.[5]

DXA has been considered the gold standard for body composition measurements.[16] Nevertheless, this imaging technique for assessing adipose tissue distribution is expensive and not feasible for routine community screening. In our results, we observed a close relationship between insulin resistance and dysfunctional VAT. Interestingly, men had higher amounts of VAT than women. Meanwhile, women and men with ≥VAT median had similar TyG values (data not shown). Moreover, the ROC curves indicate a moderate predictive ability of TyG to discriminate VAT in women (AUC = 0.713), but it was weak for men (AUC = 0.570).

The connection between body fat distribution and adipose-tissue biology with insulin resistance varies by sex, age and other factors.[11] In general, women have more total body fat mass and men present with higher abdominal/visceral fat mass.[11] However, decreased levels of oestrogen and adipose tissue redistribution by increased depots of VAT are characterized in postmenopausal women.[11,12] A disbalance of hormonal levels promotes insulin resistance and an atherogenic lipid profile, which increases the risk of CVD in older women.[11] Interestingly, some studies have suggested that obese women are more insulin sensitive than men despite a higher amount of VAT;[12] however, the mechanism is still unclear. Recently, the Netherlands Epidemiology of Obesity Study showed that in obese women, VAT was differently associated with cardiometabolic risk factors as compared with obese men.[57] However, this outcome was in contrast with Ferrara *et al.*, who reported that older obese men are more insulin resistant compared with older women, even adjusted for differences in abdominal fat distribution measured by DXA.[13] One possible explanation for our results could be that women exhibit a greater amount of FFA delivery derived from VAT lipolysis.[58] Moreover, Serra *et al.* showed that postmenopausal women (overweight or obese) diagnosed with MetS had lower adipose-tissue lipoprotein-lipase activity and limited capacity for lipid accumulation in subcutaneous abdominal adipose tissue, leading to higher levels of lipids, accumulation of VAT and insulin resistance.[59]

Our results reinforce that a VAT dysfunction and higher TyG values increase risk of developing

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NAFLD and suggest a role for glucose intolerance. Moreover, the ROC analysis reflected that TyG could be a suitable predictor of VAT. Chronic diseases are the leading causes of death and disability worldwide. MetS comprises several clinical and metabolic risk factors that increase the risk of developing T2DM and other comorbidities.[1] Individuals with T2DM and NAFLD exhibit more severe insulin resistance and liver damage. Also, in T2DM the presence of fatty liver is associated with poor glycaemic control, resulting in the need for higher insulin doses.[5] Meanwhile, ageing and biological differences between men and women play an important role in body fat distribution and health status. Our findings suggest a strong association between excessive accumulation of VAT, insulin resistance, cardiometabolic risk factors and poor liver status in subjects with MetS. Moreover, the TyG, a novel marker of insulin resistance could be used as an easy and reliable marker for dysfunctional VAT, which could constitute a new proxy for healthcare professionals in the screening of individuals diagnosed with MetS. In this context, the improvement of knowledge of these inter-relationships in subjects with MetS should be useful in easily identifying individuals with a high risk of NAFLD, which may allow early intervention and prevention of NAFLD complications.

The strengths of this study are that VAT was objectively measured with a validated imaging technique. Also, the novelty of this study comes from the use of TyG as a suitable marker of VAT in subjects at high cardiovascular risk diagnosed with MetS. However, some limitations require consideration. First, there is the relatively small sample size. Despite this, the achieved statistical power for VAT and TyG variables was higher than 90%. Second, the cross-sectional design cannot imply a causal relationship. Third, there is lack of NAFLD diagnosis by liver biopsy or imaging techniques, but important to note that liver biopsy is not available or feasible in large epidemiological studies. On the other hand, we used validated non-invasive markers to estimate hepatic fat accumulation.[38]

Conclusion

VAT and the TyG were associated with liver and cardiometabolic risk factors linked to NAFLD in individuals with overweight/obesity and MetS. Moreover, we demonstrated that in addition to anthropometric measurements or the DXA approach, TyG could be a useful simple marker to identify dysfunctional VAT phenotype in patients with diabetic profiles and MetS manifestations.

Author contribution(s)

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Itziar Abete: Conceptualization; Formal analysis; Investigation; Writing-original draft; Writing-review & editing.

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CHAPTER 3

Influence of lifestyle factors and staple foods from the Mediterranean diet on non-alcoholic fatty liver disease among older individuals with metabolic syndrome features

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Applied nutritional investigation

Influence of lifestyle factors and staple foods from the Mediterranean diet on non-alcoholic fatty liver disease among older individuals with metabolic syndrome features

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A R T I C L E   I N F O

A B S T R A C T

Objective: Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver morbidity. This condition often is accompanied by obesity, diabetes, and metabolic syndrome (MetS). The aim of this study was to evaluate the connection between lifestyle factors and NAFLD in individuals with MetS.
Methods: A cross-sectional study with 328 participants (55−75 y of age) diagnosed with MetS participating in the PREDIMED-Plus trial was conducted. NAFLD status was evaluated using the non-invasive hepatic steatosis index (HSI). Sociodemographic, clinical, and dietary data were collected. Adherence to the Mediterranean diet (mainly assessed by the consumption of olive oil, nuts, legumes, whole grain foods, fish, vegetables, fruits, and red wine) and physical activity were assessed using validated questionnaires.
Results: Linear regression analyses revealed that HSI values tended to be lower with increasing physical activity tertiles (T2, β = −1.47; 95% confidence interval [CI], −2.73 to −0.20; T3, β = −1.93; 95% CI, −3.22 to −0.65 versus T1, P_{trend} = 0.001) and adherence to the Mediterranean diet was inversely associated with HSI values: (moderate adherence β = −0.70; 95% CI, −1.92 to 0.53; high adherence β = −1.57; 95% CI, −3.01 to −0.13 versus lower, P_{trend} = 0.041). Higher tertiles of legume consumption were inversely associated with the

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highest tertile of HSI (T2, relative risk ratio [RRR], 0.45; 95% CI, 0.22−0.92; *P* = 0.028; T3, RRR, 0.48; 95% CI, 0.24−0.97; *P* = 0.041 versus T1).

Conclusion: Physical activity, adherence to the Mediterranean diet, and consumption of legumes were inversely associated with a non-invasive marker of NAFLD in individuals with MetS. This data can be useful in implementing precision strategies aimed at the prevention, monitoring, and management of NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become a prevalent chronic liver disease, as it is the primary cause of liver-related morbidity and mortality [1]. The increasing rates of NAFLD most likely accompany the increase in incidence of obesity, type 2 diabetes, metabolic syndrome (MetS), and cardiovascular disease (CVD) [2,3], especially in Western countries [1]. Indeed, NAFLD is a multifactorial chronic condition whose pathogenesis results from a complex interaction among genes, gut microbiota, and lifestyle factors [4]. Furthermore, the aging process is associated with an increased risk for developing cardiometabolic abnormalities and NAFLD progression [5]. NAFLD encompasses a spectrum of liver damage features being characterized at the initial stage by an excessive accumulation of intrahepatic triacylglycerols, which can progress to non-alcoholic steatohepatitis (NASH) and eventually lead to cirrhosis, hepatocellular carcinoma (HCC), or both if not detected early and treated [4]. Liver biopsy is the gold standard for NAFLD diagnoses [4]. However, it is an expensive and invasive procedure that may result in clinical complications [4]. Thus, several alternative non-invasive liver scores have been devised and developed [2−4]. The hepatic steatosis index (HSI) has demonstrated good performance in several population studies and has been used for large-scale NAFLD primary screening [6−8]. The management of all stages of NAFLD has been focused on improving the metabolic profile by encouraging a healthy lifestyle, such as adherence to certain dietary patterns and increased physical activity [2−4]. The Mediterranean diet (MedDiet) is a healthy dietary pattern that includes high consumption of plant-derived foods (fruits, vegetables, and legumes), whole grain foods, fish, olive oil, nuts, and low to moderate intake of red wine, meat, and dairy products [9]. Additionally, the MedDiet has demonstrated beneficial effects on the lipid profile, glycemic control, and blood pressure [10]. The presence of these clinical conditions are associated with higher risk for NAFLD and more advanced disease stages [2]. Epidemiologic and clinical studies have suggested that staple components of the MedDiet provide specific health bioactive compounds with healthy antioxidant and anti-inflammatory properties [10−13]. In fact, the effects of the MedDiet on liver status could be attributed to specific compounds such as polyphenols, fiber, carotenoids, ω-3 polyunsaturated fatty acid (PUFA), and oleic acid [12,14]. Physical activity has been shown to potentially reduce hepatic steatosis and improve insulin resistance, some MetS features, and cardiovascular events [15]. Current available recommendations suggest weight loss for NAFLD treatment (−5% or −10% of initial body weight) as the key intervention based on energy restriction. However, not only is the loss of body weight important but the characteristics of nutrient composition as well as adherence to a healthy lifestyle should be strongly considered in treatment of this disease [4]. To our knowledge, there is little available data regarding lifestyle factors of older patients with MetS. Against this background, we hypothesized that lifestyle factors, especially adherence to the MedDiet and nutritional/food characteristics and physical activity, would be associated with a decreased risk for NAFLD in a population of aduklts 55 to 75 y of age diagnosed with MetS at high cardiovascular risk.

Methods

Study population and design

The PREDIMED-Plus study is a multicenter randomized trial designed to investigate the effect on CVD morbidity and mortality reduction. A detailed protocol of the study methods and population characteristics has been published previously [16]. In brief, the study recruited 6874 individuals from 23 centers located in Spain. Participants enrolled had to meet the following inclusion criteria: men ages 55 to 75 y and women ages 60 to 75 y with a body mass index (BMI) ≥27 and <40 kg/m² fulfilling at least three criteria for the MetS [17]. We excluded those individuals who self-declared the following: therapy with immunosuppressive drugs, cytotoxic agents, or systemic corticosteroids; liver injury at the time of recruitment (cirrhosis or liver failure); history of inflammatory bowel disease, alcohol abuse, or addiction, among others. Participants were randomly assigned 1:1 into two equally sized groups: an intervention group (an intensive program of weight loss based on an energy-restricted MedDiet, physical activity promotion, and behavioral support) or a control group (an energy-unrestricted MedDiet). This clinical trial was registered and conducted in accordance with the Declaration of Helsinki ethical disclosure and further guidelines. All participants signed an informed consent to participate at the beginning of the intervention trial.

This was a cross-sectional substudy with baseline data of participants from the Navarra-Nutrition Center. The sample size was calculated to find a correlation coefficient with an 80% statistical power between adherence to the MedDiet and hepatic steatosis (*r* = 0.20) considering a type I error of 5% and type II error of 10%. In all, 422 participants were registered in the preinclusion period. Of these, we excluded 2 individuals who did not meet inclusion criteria and 89 who declined participation or for other reasons, thus leaving 331 individuals for inclusion. Of these, 328 had valid data for the non-invasive liver score calculation, which is a number that has been shown suitable in comparable studies [18,19].

Study measurements

Dietary assessment

At baseline, a 143-item food frequency questionnaire (FFQ) was administered by trained dietitians in a face-to-face manner to estimate dietary intake over the previous year. The food frequency questionnaire was previously validated in a Spanish population [20]. To evaluate adherence to the MedDiet, a score based on nine dietary components was applied, as described elsewhere [21,22]. For beneficial components (vegetables, fruits and mixed nuts, legumes, cereals, fish and seafood), participants were assigned a value of 0 for consumption below the component sex-specific median and consumption above the median was assigned a value of 1, as was fat intake considering the ratio of monounsaturated fatty acid to saturated fatty acid. For components presumed to be detrimental (meat and dairy products), individuals were assigned a value of 1 for consumption below the component sex-specific median and 0 for consumption above the median. For the alcohol component, a value of 1 was assigned to men consuming 10 to <50 g/d and women consuming 5 to <25 g/d and 0, otherwise. Thus, the total MedDiet punctuation ranged from 0 (*minimum adherence*) to 9 (*maximum adherence*). MedDiet adherence was categorized into low (0−3 points), moderate (4−5 points), or high (6−9 points) adherence for analytical purposes [22].

Physical activity assessment

Physical activity was assessed using the short REGICOR (Registre Gironi del Cor), which was validated in Spanish population [23]. As described previously [24], this questionnaire evaluated the total energy expenditure in leisure-time physical activity (metabolic equivalent [MET]-min/wk) considering light (<4 MET), moderate (4−5.5 MET), and vigorous (≥6 MET) physical activity. Also, the number of weekly hours of sedentary behavior was considered [25]. For this study, physical activity was expressed as MET-h/wk and categorized by tertiles.

Sociodemographic, lifestyle, and clinical variables

At baseline, data on sociodemographics, lifestyle, history of illnesses, and medication use were collected during the personal interview with standardized questionnaires. Smoking status was categorized as never, former, or current smoker. Trained dietitians measured weight and height using calibrated equipment following the PREDIMED-PLUS standardized protocol [16]. BMI was calculated as the body weight divided by the squared height (kg/m²). Determinations of fat mass (total, trunk, android, gynoid, and visceral) were performed using dual-energy x-

ray absorptiometry (Lunar iDXA, Madison, WI, USA; connected with enCore software, version 6) by trained personnel following the instructions of the equipment as described elsewhere [26]. Overnight fasting blood was collected. Serum and plasma samples were immediately frozen at $-80°C$. Biochemical variables, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG), glucose, and hemoglobin A1c (HbA1c), were determined with specific kits according to manufacturers' protocols, as previously described [26,27]. Low-density lipoprotein cholesterol (LDL-C) and very-low-density lipoprotein cholesterol (VLDL-C) were calculated using the Friedewald formula and TGs/5, respectively [28]. The TG-glucose index (TyG index) was estimated as the logarithm of fasting TG (mg/dL) × fasting glucose (mg/dL)/2 [29]. MetS status was defined when three or more of the following alterations were clinically ascertained [17]: 1) To have a waist circumference ≥102 cm in men and ≥88 cm in women. 2) To have elevated TG levels ≥150 mg/dL or drug treatment for hyperlipidemia. 3) To have reduced HDL-c <40 mg/dL in men and <50 mg/dL in women or drug treatment. 4) To have elevated systolic blood pressure ≥130 mmHG and/or diastolic blood pressure ≥85 mmHg or current use of antihypertensive medication. 5) To have elevated fasting glucose ≥100 mg/dL or drug treatment. According to guidelines from the International Diabetes Federation/National Heart, Lung and Blood Institute/American Heart Association (2009) [17]. Diabetes was diagnosed as described in the American Diabetes Association guidelines [30].

Non-invasive liver score assessment

The non-invasive HSI has been reported as a useful screening tool with valuable accuracy predictions of NAFLD [6,7] validated in a large group of individuals [8]. The HSI considers the AST-to-ALT ratio, BMI, presence of diabetes mellitus, and sex (women), as follows:

$$HSI = 8 * ALT/AST + BMI + (+2, \text{ if type 2 diabetes, 0 otherwise})$$
$$+ (+2, \text{ if female, 0 otherwise}) [8].$$

The lack of primary or secondary causes of hepatic fat accumulation were considered as described by American Association for the Study of Liver Diseases [4].

Statistical analyses

Continuous variables are presented as mean and (95% confidence interval [CI]), whereas categorical variables as counts (n) and frequencies (%). Categorical data were analyzed by the χ^2 test. The cohort study was stratified into HSI tertiles based on sex; HSI for men: T1 (≤40), T2 (>40 to <43.7), T3 (≥43.7 to ≤54.8); HSI for women: T1 (≤41), T2 (>41 to <46), T3 (≥46 to 57.4). Baseline characteristic differences among groups were analyzed by analysis of variance. The associations between HSI and other variables were fitted by analysis of covariance after adjusting for age, total energy intake, and alcohol intake as continuous variables with Bonferroni correction for multiple comparisons. To examine the association between HSI and lifestyle variables (physical activity across tertiles and MedDiet

adherence), we applied linear regression analyses, both performed after adjustment in model 1 for age. Further adjustments for energy intake, alcohol consumption, smoking status, high blood pressure, or antihypertensive medication were accordingly applied in model 2. Model 3A was further adjusted for MedDiet adherence and model 3B for MedDiet and physical activity in tertiles (MET-h/wk): T1 (0 to 22.5) as reference; T2 (>22.5 to ≤61.4); T3 (>61.4 to 321.7). MedDiet score was stratified according to adherence: low (0−3 points) as reference; moderate (4−5 points); high (6−9 points). To calculate P_{trend}, the physical activity and MedDiet adherence were treated as continuous variables. A linear regression analysis was carried out to evaluate the relationship between legume consumption and HSI. Furthermore, a multinomial logistic regression analysis was performed to investigate the association of legume consumption categorized in tertiles T1 (≤16.1 g/d), T2 (>16.1 to ≤20.8 g/d), and T3 (>20.8 g/d) with the HSI as dependent variable categorized in tertiles, after adjusting for potential confounders. The analysis in model 1 was adjusted for age, smoking status, energy intake, and alcohol consumption. Model 2 was further adjusted for physical activity and TGs. The effect was estimated using the relative risk ratio (RRR) with 95% CI. Analyses were carried out with Stata software version 12 (StataCorp LP, College Station, TX, USA). *P*-values are two tailed; $P < 0.05$ was considered statistically significant.

Results

Participants' characteristics

No significant differences were observed in the mean age among groups (Table 1). Participants included in the 3rd tertile of the HSI registered higher BMI and waist circumference than the rest of participants. Also, a worse glucose metabolism control was observed in these group of participants (3rd tertile) with significantly higher glucose, TyG index, a higher frequency of diabetics (all p <0.001), and HbA1c (p = 0.001). No significant differences were observed in blood pressure (systolic and diastolic) and smoking habit among tertiles (Table 1).

HSI, lipid profile, and body composition

Lipid profile and body composition variables are described in Table 2. Tertile 3 group exhibited higher levels of TGs (mean 161.9 mg/dL; 95% CI, 149.9−173.8), VLDL-C (mean 32.4 mg/dL; 95% CI, 30−34.8), serum levels, and the TG-to-HDL-C ratio (mean 3.9; 95% CI, 3.5−4.3) compared with T1 (all *P* < 0.05). Nevertheless, individuals in T2 exhibited higher TC levels than those in T1. Also, LDL-C and HDL-C levels did not differ significantly among tertiles

Table 1
Main characteristics of patients diagnosed with MetS according to HSI tertiles

	Total	T1	T2	T3	*P*-value
	(N = 328)	(n = 110)	(n = 109)	(n = 109)	
Men, n (%)	180 (54.9)	60 (54.6)	60 (55.1)	60 (55.1)	0.996
Age, y	65.8 (65.2−66.4)	66.2 (65.2−67.1)	66.3 (65.4−67.3)	64.9 (64−65.9)	0.082
BMI, kg/m²	32.2 (31.8−32.5)	29.5 (29.1−29.9)	31.7 (31.3−32.1)	35.3 (34.8−35.7)	<0.001
Weight, kg	86.1 (84.8−87.4)	79.6 (77.6−81.5)	84.4 (82.4−86.3)	94.3 (92.4−96.3)	<0.001
Waist circumference, cm	107.1 (106.1−108.1)	101.4 (100−102.8)	106.1 (104.7−107.5)	113.8 (112.4−115.2)	<0.001
Glucose, mg/dL	119.3 (115.7−123.0)	108.5 (102.4−114.5)	119.6 (113.6−125.6)	130.1 (124.1−136.2)	<0.001
HbA1c, %	6.1 (6−6.2)	5.9 (5.7−6)	6.2 (6−6.4)	6.3 (6.1−6.5)	0.001
TyG index	9 (8.9−9)	8.8 (8.7−8.8)	9 (8.9−9.1)	9.2 (9.1−9.3)	<0.001
SBP, mm Hg	142 (140.2−143.6)	141.2 (138.3−144.2)	143.6 (140.6−146.6)	140.9 (137.9−143.9)	0.390
DBP, mm Hg	86.2 (85.3−87.2)	84.9 (83.2−86.5)	86.3 (84.7−88)	87.5 (85.5−89.1)	0.093
Diabetes, n (%)	125 (38.1)	22 (20)	46 (42.2)	57 (52.3)	<0.001
High blood pressure or hypertensive medication, n (%)	318 (97)	104 (94.6)	106 (97.3)	108 (99.1)	0.145
Smoking status, n (%)					
Never	133 (40.6)	52 (47.3)	43 (39.5)	38 (34.9)	0.134
Former	154 (47)	44 (40)	49 (45)	61 (56)	
Current	41 (12.5)	14 (12.7)	17 (15.6)	10 (9.2)	
Alcohol intake, g/d	12 (10.1−13.8)	9.7 (6.6−12.9)	12.6 (9.5−15.8)	13.5 (10.3−16.7)	0.221
HSI, arbitrary units	43.1 (42.5−43.6)	38.1 (37.6−38.5)	42.6 (42.2−43.1)	48.5 (48.1−49)	<0.001

BMI, body mass index; DBP, diastolic blood pressure; HbA1C, hemoglobin A1c; HSI, Hepatic Steatosis Index; MetS, metabolic syndrome; TyG index, triacylglyceride and glucose index; SBP, systolic blood pressure
Data are presented as mean (95% CI) and frequencies (%); *P* < 0.05 considered as statistically significant

Table 2
Lipid profile, DXA estimation, and lifestyle information according to HSI tertiles in patients with MetS

	T1	T2	T3	P-value
	(n = 110)	(n = 109)	(n = 109)	
Lipid profile				
Total cholesterol, mg/dL	194.7 (187.8−201.5)*	207 (200.1−213.8)	202.4 (195.5−209.3)	0.045
LDL-C, mg/dL	123.2 (117−129.5)	131.1 (124.8−137.5)	125.9 (119.6−132.2)	0.212
HDL-C, mg/dL	46.4 (44.5−48.4)	47.4 (45.4−49.3)	45.2 (43.2−47.1)	0.291
TG, mg/dL	128.5 (116.6−140.4)†	149 (137−160.9)	161.9 (149.9−173.8)	<0.001
TG/HDL-C ratio	3 (2.6−3.3)†	3.5 (3.1−3.9)	3.9 (3.5−4.3)	0.004
VLDL-C, mg/dL	25.7 (23.3−28.1)†	29.8 (27.4−32.2)	32.4 (30−34.8)	<0.001
DXA estimation‡				
Total fat, kg	28.7 (27.5−29.9)*,†	32.9 (31.7−34.1)§	40.1 (38.8−41.3)	<0.001
Trunk fat, kg	17.3 (16.6−18.1)*,†	20.1(19.4−20.8)§	24.4 (23.7−25.1)	<0.001
Android fat, kg	3.1 (2.9−3.2)*,†	3.6 (3.5−3.8)§	4.4 (4.3−4.6)	<0.001
Gynoide fat, kg	4.1 (3.9−4.4)*,†	4.7 (4.4−4.9)§	5.9 (5.6−6.1)	<0.001
Visceral fat, kg	2.0 (1.8−2.1)*,†	2.4 (2.2−2.5)§	2.8 (2.6−3)	<0.001
Lifestyle variables				
Physical activity (MET-h/wk)	58.5 (50.4−66.6)†	51.4 (43.3−59.6)	41.1 (32.9−49.3)	0.014
MedDiet Score (0−9)	4.7 (4.4−5)†	4.4 (4.1−4.7)	4.1 (3.8−4.4)	0.015

DXA, dual-energy x-ray absorptiometry; HDL-C, high-density lipoprotein cholesterol; HSI, Hepatic Steatosis Index; LDL-C, low-density lipoprotein cholesterol; MedDiet, Mediterranean diet; MET, metabolic equivalent; MetS, metabolic syndrome; TG, triacylglycerol; VLDL-C, very-low-density lipoprotein cholesterol.
$P < 0.05$ considered statistically significant. Data expressed as mean (95% CI). Values adjusted for age, total energy intake, and alcohol intake as continuous covariates
*Significant differences between T1 and T2
†Significant differences between T1 and T3
§Significant differences between T2 and T3
‡DXA measurements available for 268 patients (T1 = 85; T2 = 92; T3 = 89), visceral fat available for 252 patients (T1 = 81; T2 = 88; T3 = 83).

(Table 2). Indeed, individuals from T3 HSI had a higher total, trunk, android, gynoid, and visceral fat mass than those from T2 and T1 ($P < 0.05$).

Relationship between HSI and lifestyle variables

Lifestyle variables such as physical activity and adherence to the MedDiet tended to decrease with increasing HSI tertiles, with significant differences between T1 and T3 (Table 2). The association between HSI, physical activity, and adherence to the MedDiet (Table 3) revealed that participants in T2 of physical activity had a significant 1.47 lower units of HSI (95% CI, −2.73 to −0.20), whereas higher levels of physical activity (T3) were associated with 1.93 lower units of HSI (95% CI, −3.22 to −0.65; P_{trend} = 0.001).

Moreover, the change of the HSI according to adherence to the MedDiet was −0.70 units (95% CI, −1.92 to 0.53) for moderate adherence, and −1.57 units (95%CI, −3.01 to −0.13) for high adherence (P_{trend} = 0.041). The daily consumption of each component of the MedDiet was also assessed according to HSI tertiles (Table 4) and no statistical differences were noted among most food groups. Interestingly, the lowest tertile group of the HSI reported a significant higher consumption of legumes (mean 21.6 g/d; 95% CI, 20−23.2) and a lower total intake of meat (mean 144 g/d; 95% CI, 134.8−153.1) than the highest tertile group (Table 4). Some statistical associations were found concerning meat consumption, which were not confirmed in adjusted analyses. A linear regression analysis demonstrated a negative relationship (Fig. 1) between HSI and

Table 3
Linear regression analyses model, exploring the association between physical activity and MedDiet adherence (as independent factors with the HSI as dependent factor) in patients with MetS

						R^2 adjusted	P_{trend}
Physical activity (MET-h/wk)							
	T1 (0−22.5)	T2 (>22.5−≤61.4)		T3 (>61.4−321.7)		R^2 adjusted	P_{trend}
		Regression coefficient	95% CI	Regression coefficient	95% CI		
Crude	0 Ref.	−1.51	(−2.79 to −0.23)	−2.24	(−3.52 to −0.96)	0.031	<0.001
Model 1	0 Ref.	−1.46	(−2.74 to −0.19)	−2.11	(−3.39 to −0.83)	0.037	<0.001
Model 2	0 Ref.	−1.54	(−2.81 to −0.27)	−2.11	(−3.39 to −0.83)	0.047	<0.001
Model 3A	0 Ref.	−1.47	(−2.73 to −0.20)	−1.93	(−3.22 to −0.65)	0.057	0.001
Mediterranean diet adherence (0−9 points)							
	Low (0−3)	Moderate (4−5)		High (6−9)		R^2 adjusted	P_{trend}
		Regression coefficient	95% CI	Regression coefficient	95% CI		
Crude	0 Ref.	−1.08	(−2.31 to 0.15)	−1.89	(−3.32 to −0.45)	0.015	0.010
Model 1	0 Ref.	−0.92	(−2.16 to 0.31)	−1.83	(−3.27 to −0.40)	0.024	0.014
Model 2	0 Ref.	−0.72	(−1.97 to 0.52)	−1.88	(−3.33 to −0.43)	0.033	0.016
Model 3B	0 Ref.	−0.70	(−1.92 to 0.53)	−1.57	(−3.01 to −0.13)	0.061	0.041

HSI, Hepatic Steatosis Index; MedDiet, Mediterranean diet; MET, metabolic equivalent; MetS, metabolic syndrome.
Model 1: Adjusted for age, as continuous covariate.
Model 2: Adjusted for age, energy intake, and alcohol consumption as continuous covariates and smoking status and high blood pressure or taking treatment as categorical covariates.
Model 3A: Model 2 + MedDiet adherence as continuous covariate.
Model 3B: Model 2 + physical activity as continuous covariate.

Table 4
Food group and dietary intake according to Hepatic Steatosis Index tertiles (HSI) in subjects with metabolic syndrome

	T1	T2	T3	P value
	(n = 110)	(n = 109)	(n = 109)	
Energy, macronutrients and fiber intake				
Total energy (Kcal/d)	2606 (2511−2702)	2559 (2463−2655)	2610 (2514−2706)	0.717
Carbohydrate (g/d)	281.1 (273.9−288.2)	284.4 (277.3−291.6)	277.9 (270.8−285.1)	0.456
Protein (g/d)	100.8 (98.2−103.4)	102.2 (99.7−104.8)	103.9 (101.3−106.5)	0.255
Lipid (g/d)	109.0 (105.8−112.2)	106.8 (103.6−110.1)	109.0 (105.8−112.3)	0.570
Monounsaturated lipids	55.5 (53.4−57.5)	54.0 (52.0−56.1)	56.0 (53.9−58.0)	0.399
Saturated lipids	26.7 (25.7−27.7)	26.4 (25.3−27.4)	27.9 (26.8−28.9)	0.103
Monounsaturated/ saturated ratio	2.2 (2.1−2.2)	2.1 (2.0−2.2)	2.1 (2.0−2.2)	0.483
Total fiber (g/d)	30.2 (28.7−31.7)	30.2 (28.6−31.7)	29.3 (27.7−30.8)	0.662
Foods and nutrient intake				
Dairy products (g/d)	361.1 (321.4−400.8)	418.0 (378.2−457.9)	383.4 (343.5−423.4)	0.137
Legumes (g/d)	21.6 (20.0−23.2)[a,c]	19.8 (18.2−21.5)	18.6 (16.9−20.2)	0.035
Meat (g/d)	144.0 (134.8−153.1)[a,c]	144.5 (135.4−153.7)[b,c]	161.4 (152.2−170.6)	0.013
Fruits (g/d)	447.2 (404.4− 490.0)	449.0 (406.0−492.0)	394.1 (350.9−437.2)	0.137
Vegetables (g/d)	333.2 (310.6−355.8)	328.0 (305.3−350.7)	332.5 (309.7−355.3)	0.942
Cereals (g/d)	201.1 (187.1−215.1)	202.5 (188.5−216.6)	198.2 (184.1−212.3)	0.909
Fish and seafoods (g/d)	102.1 (94.2−110.1)	95.6 (87.7−103.6)	98.0 (90.0−106.0)	0.516

$P < 0.05$ is considered statistically significant. Data are expressed as mean (95% CI). Values were adjusted for total age, energy intake except for energy intake and alcohol intake as continuous covariates.
[a,c] significant differences between T1 vs T3.
[b,c] significant differences between T2 vs T3.
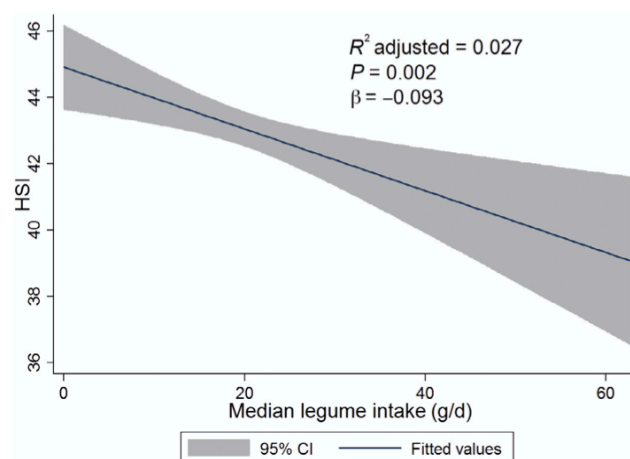
Fig. 1. Regression analysis with HSI. HSI, Hepatic Steatosis Index.

legume consumption (R^2 adjusted = 0.027; P = 0.002). Reinforcing this notion, a statistically significant inverse association between legume intake (g/d) and the highest tertile of HSI was observed. The relative risk for the HSI (T3) according to tertiles of legume consumption for the final fully adjusted model (Table 5) was as follows: 1.00 (reference); T2, 0.45 (95% CI, 0.22−0.92); T3, 0.48 (95% CI, 0.24−0.97).

Discussion

Emerging clinical data have established a close relationship between NAFLD and MetS [31]. In this cross-sectional cohort study, key components related to cardiometabolic risk factors disclosed a direct association with higher HSI values. To the best of our knowledge, this was the first study to evaluate the relationship between lifestyle factors and the specific role of typical Mediterranean foods, with NAFLD characteristics in an older population diagnosed with MetS. In particular, the HSI has been proposed as a predictor of liver steatosis [7]. The accuracy of HSI was validated in a large cohort study using ultrasonography to dignose fatty liver [8]. Cut-off values for the diagnosis of NAFLD were established such that values >36 confirmed the diagnosis of steatosis [8]. In fact, the use of non-invasive liver scores might be useful for the diagnosis and prediction of NAFLD [7,32,33]. In the present study, participants at the highest HSI tertile disclosed a pro-atherogenic lipid profile. Additionally, they had higher blood glucose levels and disrupted insulin homeostasis as assessed by TyG index as an indicator of insulin resistance [29], and it could predict risk for NAFLD [34]. These findings may be explained by the involvement of glucose and insulin in the activation of several pathways related to lipogenesis [32]. The present results are consistent with the fact that muscle and liver insulin resistance promote the accumulation of

Table 5

Multivariate analysis concerning the associations between legume consumption and NAFLD according to Hepatic Steatosis Index (HSI) in subjects featured with metabolic syndrome

| | | Hepatic Steatosis Index | | | | |
| | | T1 | T2 | | T3 | |
	n		RRR (95% CI)	P value	RRR (95% CI)	P value
Legume (g/d)						
Crude						
Tertile 1 (≤ 16.1)	139	1 Ref.	1 Ref.		1 Ref.	
Tertile 2 ($>16.1 - \leq 20.8$)	93	1 Ref.	0.89 (0.47 to 1.68)	0.713	0.51 (0.27 to 0.99)	0.046
Tertile 3 (>20.8)	96	1 Ref.	0.74 (0.39 to 1.41)	0.364	0.53 (0.28 to 0.99)	0.049
Model 1						
Tertile 1 (≤ 16.1)	139	1 Ref.	1 Ref.		1 Ref.	
Tertile 2 ($>16.1 - \leq 20.8$)	93	1 Ref.	0.94 (0.49 to 1.80)	0.846	0.50 (0.26 to 0.99)	0.048
Tertile 3 (>20.8)	96	1 Ref.	0.81 (0.42 to 1.59)	0.548	0.54 (0.27 to 1.06)	0.072
Model 2						
Tertile 1 (≤ 16.1)	139	1 Ref.	1 Ref.		1 Ref.	
Tertile 2 ($>16.1 - \leq 20.8$)	93	1 Ref.	0.85 (0.44 to 1.66)	0.638	0.45 (0.22 to 0.92)	0.028
Tertile 3 (>20.8)	96	1 Ref.	0.74 (0.37 to 1.46)	0.382	0.48 (0.24 to 0.97)	0.041

Model 1: Adjusted for age, energy intake, and alcohol consumption as continuous covariates, and smoking status as categorical covariate.
Model 2: model 1 + triacylglycerols and physical activity as continuous covariates.
Abbreviations: HSI, Hepatic Steatosis Index; RRR, relative risk ratio.

several lipid metabolites and impair VLDL assembly and secretion. The overproduction of VLDL particles leads to an increased free fatty acid flux into plasma, which augments the risk for liver steatosis [32,35]. Additionally, there are several clinical studies confirming that visceral adipose tissue induces insulin resistance, inflammation, and liver damage [35–37]. In the present research, visceral adiposity increased across tertiles of the HSI, concurring with the observation of a strong association between visceral adipose tissue and fatty liver infiltration [36,37].

Some investigations have demonstrated the effectiveness of physical activity in the prevention and management of chronic diseases [38]. In the present study, the highest tertile of physical activity ($>61.4-321.7$ MET-h/wk) showed a lower HSI. In agreement with our results, some studies indicated that physical activity could attenuate or delay NAFLD progression [15,39,40]. A recent analysis of PREDIMED-PLUS data indicated that moderate vigorous physical activity was inversely associated with cardiometabolic risk factors such as abdominal obesity and low HDL-C as independent components of the MetS [24]. Furthermore, higher physical activity was inversely related to NAFLD and participants who had a physical activity ≥ 500 MET-min/wk showed a 34% decreased risk for NAFLD compared with sedentary individuals [39]. In fact, physical inactivity and lower aerobic fitness could play a key role in mechanisms related to fat regulation and mitochondrial dysfunction [40]. It is important to highlight that physical activity is a modifiable risk factor, which might have a protective effect on liver status. Several mechanisms for the effects of physical activity on NAFLD have been proposed, but duration or the influence of the type of exercise treatment remains unclear [15].

Few intervention studies have explored the associations between the MedDiet and NAFLD [13,18,19,41]. However, specific components consumed in the context of the MedDiet have shown enough scientific evidence based on epidemiologic clinical trials and animal studies on features of CVD and MetS [11,14]. This healthy dietary pattern provides nutrients and bioactive compounds with antioxidant capacity and anti-inflammatory effects [10,11,33,42]. The MedDiet pattern is characterized by a high consumption of fruit, vegetables, non-refined cereals, legumes, unsaturated fatty acids (olive oil and nuts); moderate intake of fish, seafood, fermented dairy products, poultry, and eggs; low-to-moderate amounts of wine, and low consumption of red meat, processed meat, and sweets [9]. The consumption of most of the

healthful components of the MedDiet is associated with an improvement of the serum lipid profile, insulin resistance, liver enzymes, and other factors linked to NAFLD [13,18,43,44]. According to the present data, high adherence to the MedDiet was inversely and significantly associated with the HSI after adjusting for potential confounders. Such findings are consistent with a previous study showing that MedDiet ameliorated hepatic steatosis and improved insulin sensitivity [13,18]. In contrast, Kontogianni et al. did not find differences in the adherence to the MedDiet between individuals diagnosed with NAFLD and healthy individuals [19]. In fact, the authors suggested that non-dietary factors have a strong effect on pathogenesis and development of this disease [19].

The association between light to moderate alcohol consumption and the severity and pathogenesis of NAFLD is still controversial [45]. Nevertheless, moderate alcohol consumption might improve insulin sensitivity and CVD mortality [45]. Ajmera et al. suggested that in individuals diagnosed with NAFLD without NASH, the cardiovascular benefits of moderate alcohol consumption could have been outweighed by injurious effects on liver status [45]. Moreover, modest wine consumption could reduce prevalence of suspected NAFLD (higher levels of ALT) in patients at high risk for coronary heart disease [46]. However, further high-quality, clinical studies are needed to better clarify the effects of moderate alcohol consumption on liver health, NASH histology, and NAFLD severity. When we recalculated the MedDiet adherence score without considering alcohol consumption, the inverse association between the MedDiet and the HSI did not change. On other hand, we noted that legume consumption decreases across tertiles of HSI. In fact, when our participants were stratified according to legume consumption tertiles, an apparent inverse association was found with the highest HSI values. Furthermore, we also observed that higher consumption of legumes was associated with 52% lower odds to be in the top HSI tertile, even after controlling for potential dietary and non-dietary confounders. These results are consistent with those of previous clinical studies that evaluated the influence of legume intake on obesity and metabolic disorders [47,48]. The PREDIMED study prospectively found that greater legume intake (28 g/d) was associated with a lower risk for type 2 diabetes in patients at high risk for CVD [48]. Several authors have claimed that the beneficial effects of legume intake are attributed to the presence of vegetable protein, fiber, antioxidants, phytochemicals, and other bioactive compounds [47]. Legumes are particularly rich in fiber (soluble fiber and resistant starch) that might exert effects on digestibility and lowering

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absorption rates of carbohydrates, thereby improving glycemic control [47]. Moreover, a hypolipidemic effect of legumes has been observed promoting a reduction of intestinal fat absorption and bile acid uptake thus inducing a reduction of free fatty acids and cholesterol in the liver [49]. In this regard, those with greater legume intake presented a significantly lower risk for higher HSI values. This suggests that legume consumption could ameliorate metabolic disorders related to NAFLD in patients with MetS.

Only a few clinical studies have investigated the relationship between meat consumption and NAFLD risk [12,50]. The link between meat intake and risk for developing NAFLD and comorbidities may rely on harmful meat components such as SFAs and heme-iron [14]. However, our findings showed no differences in SFA intake among HSI tertiles. Indeed, when a multivariable analysis was fully adjusted, the relationship between total meat intake and HSI values was not statistically significant. This outcome may be attributed to differences in meat subtypes [51]. Thus, Zelber-Sagi et al. indicated that meat consumption, especially red and processed meat, was independently associated with the increased risk for developing NAFLD and insulin resistance [50]. In contrast, a recent meta-analysis of observational studies reported an inverse association between white meat intake and MetS [51]. It is also important to highlight that red meat, beef internal organs, and processed meat contain more heme-iron than white meat [52]. More studies will be warranted to evaluate the role of specific meat subtypes in NAFLD.

The strengths of this analysis included the fact that it was the first study to use a representative and relatively large sample of older individuals diagnosed with MetS within the PREDIMED-Plus cohort. Additionally, the study explored the potential association between modifiable lifestyle factors and NAFLD assessed by a non-invasive liver score used for larger-scale screening studies [6,7]. However, the present research had some limitations. First, was its cross-sectional and non-prospective design. Second, liver content was not directly measured. However, we used a validated non-invasive liver marker suitable for use in clinical practice as an alternative to imaging methods or liver biopsy. Third, the study sample was made up of older white individuals diagnosed with MetS. This status limits the extrapolation of our results to other populations, although it concerns patients at increased cardiometabolic risk that abound in all Western countries.

Conclusions

This study suggested that lifestyle modifications focused on physical activity and fostering adherence to the MedDiet in older adults diagnosed with MetS might exert beneficial effects on liver status. Moreover, some foods such as legumes may play a beneficial role in the improvement of hepatic steatosis reducing the risk for NAFLD. The present findings support the recommendation of lifestyle changes (nutrition and physical activity) as a cornerstone for the prevention and precise management of NAFLD in patients with MetS.

Conflicts of Interest

None of the authors reported a conflict of interest.

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CHAPTER 4

Urinary resveratrol metabolites output: differential associations with cardiometabolic markers and liver enzymes in house-dwelling subjects featuring metabolic syndrome

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Article

Urinary Resveratrol Metabolites Output: Differential Associations with Cardiometabolic Markers and Liver Enzymes in House-Dwelling Subjects Featuring Metabolic Syndrome

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Abstract: Metabolic syndrome (MetS) components are strongly associated with increased risk of non-alcoholic fatty liver disease (NAFLD) development. Several studies have supported that resveratrol is associated with anti-inflammatory and antioxidant effects on health status. The main objective of this study was to assess the putative associations between some urinary resveratrol phase II metabolites, cardiometabolic, and liver markers in individuals diagnosed with MetS. In this cross-sectional study, 266 participants from PREDIMED Plus study (PREvención con DIeta MEDiterránea) were divided into tertiles of total urinary resveratrol phase II metabolites (sum of five resveratrol conjugation metabolites). Urinary resveratrol metabolites were analyzed by ultra-performance liquid chromatography coupled to triple quadrupole mass spectrometry (UPLC-Q-q-Q MS), followed by micro-solid phase extraction (μ-SPE) method. Liver function markers were assessed using serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT). Moreover, lipid profile was measured by triglycerides, very-low-density lipoprotein cholesterol (VLDL-c), and total cholesterol/high-density lipoprotein ratio (total cholesterol/HDL). Linear regression adjusted models showed that participants with higher total urine resveratrol concentrations exhibited improved lipid and liver markers compared to the lowest tertile. For lipid determinations: log triglycerides (β_{T3} = −0.15, 95% CI; −0.28, −0.02, *p*-trend = 0.030), VLDL-c, (β_{T3} = −4.21, 95% CI; −7.97, −0.46, *p*-trend = 0.039), total cholesterol/HDL ratio Moreover, (β_{T3} = −0.35, 95% CI; −0.66, −0.03, *p*-trend = 0.241). For liver enzymes: log AST (β_{T3} = −0.12, 95% CI; −0.22, −0.02, *p*-trend = 0.011, and log GGT (β_{T3} = −0.24, 95% CI; −0.42, −0.06, *p*-trend = 0.002). However, there is no difference found on glucose variables between groups. To investigate the risk of elevated serum liver markers, flexible regression models indicated that total urine resveratrol metabolites were associated with a lower risk of higher ALT (169.2 to 1314.3 nmol/g creatinine), AST (599.9 to 893.8 nmol/g creatinine), and GGT levels (169.2 to 893.8 nmol/g creatinine). These results suggested that higher urinary concentrations of some resveratrol metabolites might be associated with better lipid profile and hepatic serum enzymes. Moreover, urinary resveratrol excreted showed a reduced odds ratio for higher liver enzymes, which are linked to NAFLD.

Keywords: antioxidant; inflammation; liver enzymes; metabolic syndrome; non-alcoholic fatty liver disease; resveratrol

1. Introduction

Metabolic syndrome (MetS) encompasses several clinical conditions, including central obesity, hypertension, dyslipidemia, and insulin resistance leading to an inflammatory state [1], which is frequently accompanied by liver dysfunction [2]. Many clinical studies suggested that non-alcoholic fatty liver disease (NAFLD) is the liver manifestation of MetS [2–4]. NAFLD is characterized by simple hepatic steatosis (excessive triglyceride accumulation) leading to alterations in oxidative and inflammatory pathways. This state promotes non-alcoholic steatohepatitis (NASH), subsequently

cirrhosis and hepatic carcinoma in last stages [3,5]. The prevalence of NAFLD increases with rates of obesity and type 2 diabetes mellitus (T2DM), mainly due to unhealthy lifestyle behaviors [2]. It has been suggested that insulin resistance and abnormal lipid profile were strongly involved in NAFLD pathogenesis and prognosis [3]. Hyperinsulinemia increases free fatty acid levels promoting a disrupted flux of triglycerides into hepatocytes [5–7]. In NAFLD, commonly abnormal elevation of serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) have presented [3,4]. Liver biopsy is still the gold standard for diagnosing NAFLD, but some limitations regarding high cost and invasive nature hindered it being applicable in epidemiological studies [3]. In this sense, non-invasive liver markers such as transaminases, fatty liver index (FLI) and hepatic steatosis index (HSI) are recommended in individuals with obesity and MetS as a routine work-up to identify the risk of NAFLD and subjects with worse prognosis [4]. Metabolomics is the technology that analyses metabolites in a biological system, and it has been considered as a potential omics tool to investigate the impact of nutrients, foods, and dietary patterns on human health with application in precision nutrition research [8]. Moreover, metabolite biomarkers related to dietary intake could be useful as potential non-invasive biomarkers of effects and disease risk [8].

Lifestyle interventions focused on weight loss, exercise, and a healthy diet can improve the histopathological and clinical features of NAFLD [3]. Scientific evidence suggests that the modulation of dietary components can influence NAFLD pathogenesis beyond caloric restriction [9]. In this sense, many epidemiological and clinical data support that the beneficial effects of the Mediterranean diet (MedDiet) on metabolic disturbances linked to NAFLD mainly attributed to higher consumption of bioactive compounds, such as resveratrol and anthocyanins that are present in whole-grain cereals, fruits, vegetables, healthy fatty acids, and moderate intake of wine [10–15]. Resveratrol is a member of the stilbene family that is present in several foods and plants [16,17]. The primary dietary sources are red grapes and red wine, with smaller amounts present in peanuts, berries, and dark chocolate [16,18]. After the intake, resveratrol enters the gastrointestinal tract and then the liver via the hepatic portal system, and is metabolized by phase II enzymes generating sulfate (*trans-/cis-* resveratrol 3-*O*-sulfate and 4-*O*-sulfate) and glucuronide (*trans-/cis*-resveratrol 3-*O*-glucuronide and 4-*O*-glucuronide) metabolites [19,20]. The gut microbiota can metabolize the resveratrol and conjugated metabolites into dihydro-resveratrol and lunularin [19]. Several human studies have shown that the most abundant resveratrol phase II conjugates are glucuronides and sulfate metabolites in urine and plasma samples [20,21]. Limited information has been reported regarding bioavailability and pharmacokinetics of glycosylated metabolites (piceid), derived from gut microbiota and other stilbenes (piceatannol). Pharmacokinetics studies in human showed that resveratrol is highly metabolized, but it has low bioavailability [20,22,23]. In a study used radiolabeled ^{14}C-resveratrol to evaluate the bioavailability of resveratrol intake in humans, results indicated that 70% of the resveratrol absorption was recovered in urine. Moreover, the rapid sulfate conjugation by the intestine-liver could be the principal influence of their bioavailability [22]. Moreover, the bioavailability and quantity of resveratrol metabolites can be affected by several factors leading to a significant interindividual variability [19,24,25]. Despite its low bioavailability, several studies have reported that resveratrol metabolites exert beneficial effects modulating inflammatory and oxidative pathways related to several chronic diseases, such as cancer, cardiovascular disease (CVD), T2DM, obesity, and NAFLD [21]. NAFLD, most of the studies on resveratrol, and the mechanism of action have been developed in vitro and animal rodents, which used higher resveratrol concentrations, different cell lines, and animal models that often overlap [26]. The protective effects of resveratrol on NAFLD mainly include improvements in principal risk factors, such as blood glucose and insulin levels [27], lipid metabolism [28], and liver damage [29], but results have remained inconclusive. In this regard, knowledge of the underlying effects of resveratrol metabolites on liver markers and risk of NAFLD in individuals diagnosed with MetS is still needed. Thus, our objective was to determinate the potential association between some phase II urinary metabolites of resveratrol and cardiometabolic and liver markers in individuals diagnosed with Mets. We hypothesized that high

urinary resveratrol metabolites would be associated with a favorable cardiometabolic profile and hepatic markers related to the risk of NAFLD development.

2. Results

2.1. Participant Characteristics

Baseline sociodemographic, clinical, and liver characteristics stratified by sex are summarized in Table 1. This study included subjects between 55 to 75 years old (65.8 ± 5.1 years) who were overweight or obese (32.2 ± 3.4 kg/m²). Men were more prevalent as former smokers, had higher waist circumference (109.9 ± 8.5 cm), and visceral fat mass (2850.3 ± 826.2 g) compared to women (all p-values <0.05). Moreover, men showed higher levels of physical activity (3690 ± 3101.4 Metabolic Equivalent of Task (MET)/min/week) (p > 0.001). There were no differences in taking lipid-lowering and anti-diabetic medications in between genders. Likewise, glucose, homeostatic model assessment for insulin (HOMA-IR), triglycerides, and very-low-density lipoprotein cholesterol (VLDL-c) did not differ among sexes. Nevertheless, women had higher insulin, cholesterol, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) levels in comparison to men (all p > 0.05). Concerning liver markers, ALT (31.2 ± 24.5 U/L), AST (25.2 ± 16.3 U/L), and FLI (80.4 ± 14.4) were significantly higher in men than women (all p-values > 0.05). Moreover, there was significant difference in the percentage of participants with ALT values above the upper limit normal (ULN) between men and women. Meanwhile, women had higher HSI (44.0 ± 5.1) in comparison to men (p = 0.004). Regarding dietary intake and urine resveratrol metabolites (Table 2), the intake of macronutrients did not differ significantly between genders. However, men had higher energy (2689.4 ± 534.5 kcal/d, p = 0.004) intake and polyunsaturated fatty acid (PUFA) consumption (19.1 ± 7.2 g/d, p = 0.049) compared to women. Nevertheless, women consumed more vegetables (353.0 ± 124.0 g/d, p = 0.024) and had a lower grape intake (5.8 ± 12.3 g/d, p = 0.012). Moreover, men had a much higher alcohol consumption than women (19.7 ± 19.8 g/d, p < 0.001) with statistically significant differences in total red, young red, aged red, and rose wine consumption between sexes.

Table 1. Sociodemographic, clinical and liver characteristics of study participants diagnosed with MetS by sex at baseline.

	All	Men (n = 153)	Women (n = 113)	P ¶
Age (years)	65.8 (5.1)	64.6 (5.4)	67.5 (3.9)	<0.001
BMI (kg/m²)	32.2 (3.4)	31.8 (3.1)	32.8 (3.7)	0.019
Waist circumference (cm)	107.3 (9.0)	109.9 (8.5)	103.9 (8.6)	<0.001
VAT (g)	2403.6 (888.9)	2850.3 (826.2)	1831.5 (589.7)	<0.001
SBP (mmHg)	144.8 (16.3)	144.9 (15.8)	144.6 (17.0)	0.887
DBP (mmHg)	87.5 (8.5)	87.9 (8.2)	86.9 (8.9)	0.338
Type 2 diabetes, n (%)	100 (37.6)	61 (39.9)	39(34.5)	0.373
Smoking, n (%)				<0.001
Never	105 (39.5)	29 (18.9)	76 (67.3)	
Former	124 (46.6)	97 (63.4)	27 (23.9)	
Current	37 (13.9)	27 (17.7)	10 (8.8)	
Lipid-lowering treatment	93 (56.0)	54 (57.5)	39(54.2)	0.673
Any anti-diabetic treatment	69 (25.9)	43 (28.1)	26 (23.0)	0.349
Glucose (mmol/L)	6.7 (1.9)	6.8 (2.1)	6.5 (1.6)	0.286
HbA1c (%)	6.1 (0.9)	6.1 (1.0)	6.1 (0.8)	0.721
Insulin (mU/L)	14.0 (9.0)	13.0 (7.1)	15.6 (11.0)	0.020
HOMA-IR	4.2 (3.2)	3.9 (2.4)	4.7 (4.2)	0.056
Total cholesterol (mg/dL)	200.4 (36.5)	192.2 (34.2)	211.4 (36.9)	<0.001
Triglycerides (mg/dL)	148.3 (61.8)	151.1 (69.0)	144.6 (50.6)	0.402
HDL-c (mg/dL)	45.8 (10.0)	43.0 (8.9)	49.5 (10.1)	<0.001
LDL-c (mg/dL)	125.8 (33.1)	119.8 (31.3)	133.6 (33.9)	<0.001
VLDL-c (mg/dL)	29.7 (12.4)	30.2 (13.8)	28.9 (10.1)	0.402

ALT (U/L)	28.1 (20.6)	31.2 (24.5)	23.8 (12.3)	0.004
AST (U/L)	23.7 (13.4)	25.2 (16.3)	21.8 (7.4)	0.042
GGT (U/L)	42.1 (41.1)	44.9 (41.5)	38.5 (40.3)	0.209
ALT > ULN, *n* (%) *	122 (46.0)	55 (36.0)	67 (59.8)	<0.001
AST> ULN, *n* (%) *	28 (10.5)	12 (7.8)	16 (14.2)	0.097
GGT> ULN, *n* (%) *	51 (19.3)	24 (15.9)	27 (23.9)	0.103
FLI	78.7 (15.1)	80.4 (14.4)	76.4 (15.9)	0.035
HSI	43.0 (4.9)	42.3 (4.7)	44.0 (5.1)	0.004
Physical activity (MET-min/week)	3099.9 (2757.8)	3690 (3101.4)	2301.0 (1954.8)	<0.001

Data were calculated by chi-square or student's *t*-test as appropriate. Results are expressed as mean (standard deviation). *p* for differences between sexes. Abbreviations: BMI, Body mass index; VAT, visceral adipose tissue; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin; HDL-c, high-density lipoprotein cholesterol; LDL-c, Low-density lipoprotein cholesterol; VLDL, very-low-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; FLI, fatty liver index; HSI, hepatic steatosis index; MET, metabolic equivalent task.* Upper limit of normal (ULN) range for ALT (men ≥ 30 UI/L, women ≥ 19 UI/L), AST (men ≥ 37 UI/L, women ≥ 31 UI/L), and GGT (men ≥ 60 UI/L, women ≥ 40 UI/L).

Urine resveratrol metabolites (Table 2) showed that men had higher *trans*-resveratrol-3-*O*-sulfate and *cis*-resveratrol-3-*O*-glucuronide/*cis*-resveratrol-4'-*O*-glucuronide urine levels (all *p*-values < 0.05) compared to women.

Table 2. Dietary intake and urine resveratrol metabolites in participants diagnosed with MetS by sex at baseline.

	All	Men (*n* = 153)	Women (*n* = 114)	*p* ¶
Total energy intake (kcal/d)	2608.6 (539.1)	2689.4 (534.5)	2499.1 (528.0)	0.004
Carbohydrate intake (g/d)	282.9 (75.1)	287.0 (73.8)	277.5 (76.9)	0.310
Protein intake (g/d)	102.5 (22.9)	101.3 (23.8)	104.1 (21.8)	0.330
Fat intake (g/d)	108.5 (27.1)	110.9 (26.9)	105.3 (27.2)	0.097
MUFAs (g/d)	55.3 (14.3)	56.4 (14.2)	53.9 (14.5)	0.171
PUFAs (g/d)	18.3 (7.2)	19.1 (7.2)	17.3 (7.1)	0.049
Linoleic (g/d)	15.4 (6.9)	16.1 (6.8)	14.6 (7.0)	0.075
Linolenic (g/d)	1.6 (0.8)	1.6 (0.8)	1.5 (0.8)	0.317
Omega-3 (g/d)	0.9 (0.5)	0.9 (0.5)	0.9 (0.4)	0.305
Fiber (g/d)	30.0 (9.8)	29.3 (10.1)	30.9 (9.3)	0.175
Total cholesterol (g/d)	375.7 (110.8)	382.4 (124.4)	366.5 (88.9)	0.248
Total vegetables (g/d)	333.0 (124.4)	318.2 (123.0)	353.0 (124.0)	0.024
Total fruits (g/d)	423.9 (220.4)	407.3 (221.6)	446.3 (217.8)	0.154
Grapes intake (g/d)	9.7 (21.8)	12.6 (26.4)	5.8 (12.3)	0.012
Cherries and plums (g/d)	14.4 (19.4)	14.9 (19.8)	13.6 (18.9)	0.599
Nuts intake (g/d)	15.1 (18.1)	15.6 (18.1)	14.3 (18.2)	0.566
Homemade fruit juice (mL/d)	4.1 (23.6)	5.6 (29.4)	2.0 (11.5)	0.209
Fruit juice bottle (mL/d)	12.4 (54.8)	12.7 (52.9)	12.1 (57.5)	0.931
Adherence to MedDiet (0–17 points)	8.8 (2.5)	8.8 (2.4)	9.0 (2.5)	0.484
Alcohol consumption (g/d)	12.9 (17.7)	19.7 (19.8)	3.5 (7.2)	<0.001
Total red wine (g/d)	61.4 (105.9)	91.3 (120.8)	20.8 (62.1)	<0.001

Young red wine (g/d)	56.7 (105.3)	84.1 (121.3)	19.6 (61.8)	<0.001
Aged red wine (g/d)	4.7 (25.6)	7.2 (32.5)	1.2 (9.5)	0.056
Rosé wine (g/d)	10.4 (49.8)	16.7 (64.2)	1.9 (11.9)	0.017
Moscatel wine (g/d)	0.5 (7.7)	0.8 (10.1)	0.06 (0.7)	0.429
White wine (g/d)	7.4 (33.6)	10.6 (42.4)	3.1 (14.4)	0.073
trans-resveratrol-3-*O*-glucuronide (nmol/g creatinine)	0.7 (1.6)	0.7 (0.9)	0.7 (2.2)	0.973
trans-resveratrol-4′-*O*-glucuronide (nmol/g creatinine)	171.9 (375.8)	143.7 (314.7)	210.1 (444.1)	0.154
trans-resveratrol-3-*O*-sulfate (nmol/g creatinine)	0.2 (0.5)	0.2 (0.6)	0.1 (0.2)	0.023
cis-resveratrol-3-*O*-glucuronide and *cis*-resveratrol-4′-*O*-glucuronide (nmol/g creatinine)	2.0 (5.3)	2.6 (6.0)	1.1 (4.1)	0.023

Data were calculated by student's *t*-test. Results are expressed as mean (standard deviation). *p* ¹ for differences between sexes. Abbreviations: MUFAs, monounsaturated fatty acids; PUFAs, Polyunsaturated fatty acids; MedDiet, Mediterranean diet.

2.2. Association between Total Urine Resveratrol Metabolites Concentrations, Cardiometabolic Profile, and Liver Markers

The association between total urine resveratrol metabolites and glucose metabolism markers, blood lipid, and liver markers are shown in Tables 3–5, respectively. After adjusting for covariates, there were no significant associations between total urine resveratrol metabolites and glucose metabolism markers (Table 3). Table 4 summarizes values concerning lipid metabolism. No significant associations were observed between total urine resveratrol metabolites and LDL-c and log triglyceride/HDL ratio among tertiles. Although in the adjusted model, participants in the T3 had significantly lower levels of total cholesterol compared to T1 (β_{T3} = 11.67, 95% CI, −22.21 to −1.13), but there was no significant tendency among tertiles (*p*-trend = 0.108). Interestingly, individuals in the highest tertile (T3) of total urine resveratrol metabolites had significantly lower levels of log triglycerides (β_{T3} = −0.15, 95% CI, −0.28 to −0.02), VLDL-c (β_{T3} = −4.21, 95% CI, −7.97 to −0.46), and total cholesterol/HDL ratio (β_{T3} = −0.35, 95%CI, −0.66 to −0.03) after adjustment. Regarding to liver markers (Table 5), compared with those in the first tertile, individuals in the third tertile had 2.4 significant unit decrease in the log GGT (95% CI, −0.42 to −0.06; *p*-trend = 0.002) and lower log AST (β_{T3} = −0.12, 95% CI, −0.22 to −0.02; *p*-trend = 0.011). Nevertheless, the differences of other liver markers (log ALT, HSI and FLI) between levels of urinary resveratrol metabolites were not statistically significant.

Table 3. Linear regression analysis distributed in tertiles evaluating the associations between total urine resveratrol (independent variable) and glucose metabolism markers (outcome) in participants with MetS.

	Total Urine Resveratrol Metabolites (nmol/g Creatinine)			
	T1	T2	T3	*p*-Trend
	(≤4.6)	(>4.6 to 58.1)	(>58.1 to 2481.2)	
n	89	89	88	
		β Coefficient (95% IC)	β Coefficient (95% IC)	
Glucose markers				
Glucose (mmol/L)				
Crude model	0 REF.	0.02 (−0.55, 0.60)	0.11 (−0.46, 0.69)	0.677
Adjusted model	0 REF.	0.04 (−0.56, 0.63)	0.04 (−0.55, 0.63)	0.933
HbA1c (%)				
Crude model	0 REF.	−0.07 (−0.35, 0.21)	−0.01 (−0.28, 0.27)	0.842
Adjusted model	0 REF.	−0.07 (−0.36, 0.22)	−0.04 (−0.32, 0.25)	0.982
Insulin sensitivity/resistance markers				
Insulin (mU/L)				
Crude model	0 REF.	−1.23 (−3.94, 1.48)	−1.15 (−3.86, 1.55)	0.623
Adjusted model	0 REF.	−0.22 (−2.87, 2.43)	−0.58 (−3.20, 2.03)	0.672

HOMA-IR				
Crude model	0 REF.	−0.45 (−1.44, 0.54)	−0.46 (−1.44, 0.52)	0.558
Adjusted model	0 REF.	−0.11 (−1.09, 0.87)	−0.31 (−1.28, 0.65)	0.534
HOMA-%B				
Crude model	0 REF.	−15.19 (−37.11, 6.73)	−11.00 (−32.79, 10.79)	0.682
Adjusted model	0 REF.	−11.07 (−32.44, 10.30)	−6.32 (−27.35, 14.71)	0.904
FGIR				
Crude model	0 REF.	−0.12 (−0.30, 0.06)	−0.07 (−0.25, 0.11)	0.859
Adjusted model	0 REF.	−0.14 (−0.33, 0.05)	−0.07 (−0.26, 0.11)	0.922
FIRI				
Crude model	0 REF.	−0.40 (−1.29, 0.49)	−0.42 (−1.30, 0.47)	0.558
Adjusted model	0 REF.	−0.10 (−0.98, 0.78)	−0.28 (−1.14, 0.58)	0.534

Models were adjusted for sex, age, smoking status, marital status, physical activity, energy intake, and BMI. Abbreviations: HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin; HOMA-%B, HOMA of β-cell function; FGIR, fasting glucose insulin ratio, FIRI, fasting insulin resistance index; REF, reference.

Table 4. Linear regression analysis evaluating the associations between total urine resveratrol (independent variable) and blood lipids (outcome) in participants with MetS.

	Total Urine Resveratrol Metabolites (nmol/g Creatinine)			
	T1	T2	T3	*p*-Trend
	(≤4.6)	(>4.6 to 58.1)	(>58.1 to 2481.2)	
n	89	89	88	
		β Coefficient (95% IC)	β Coefficient (95% IC)	
Blood lipids				
Total cholesterol (mg/dL)				
Crude model	0 REF.	−13.10 (−23.77, −2.43)	−13.26 (−23.96, −2.56)	0.132
Adjusted model	0 REF.	−8.93 (−19.54, 1.68)	−11.67 (−22.21, −1.13)	0.108
LDL-c (mg/dL)				
Crude model	0 REF.	−11.36 (−21.15, −1.57)	−8.21 (−18.02, 1.61)	0.502
Adjusted model	0 REF.	−9.32 (−19.13, 0.48)	−7.77 (−17.57, 2.02)	0.435
HDL-c (mg/dL)				
Crude model	0 REF.	0.36 (−2.60, 3.32)	−0.38 (−3.36, 2.59)	0.674
Adjusted model	0 REF.	1.41 (−1.51, 4.32)	0.27 (−2.63, 3.18)	0.761
Log triglyceride (mg/dL)				
Crude model	0 REF.	−0.05 (−0.18, 0.07)	−0.14 (−0.26, −0.02)	0.032
Adjusted model	0 REF.	−0.06 (−0.19, 0.07)	−0.15 (−0.28, −0.02)	0.030
VLDL-c (mg/dL)				
Crude model	0 REF.	−1.48 (−5.11, 2.15)	−3.95 (−7.60, −0.30)	0.043
Adjusted model	0 REF.	−1.70 (−5.47, 2.08)	−4.21 (−7.97, −0.46)	0.039
Log triglyceride/HDL ratio				
Crude model	0 REF.	−0.07 (−0.23, 0.09)	−0.14 (−0.30, 0.02)	0.122
Adjusted model	0 REF.	−0.10 (−0.26, 0.07)	−0.16 (−0.33, 0.002)	0.106
Total cholesterol/HDL ratio				
Crude model	0 REF.	−0.38 (−0.69, −0.08)	−0.32 (−0.62, −0.01)	0.304
Adjusted model	0 REF.	−0.39 (−0.70, −0.07)	−0.35 (−0.66, −0.03)	0.241

Models were adjusted for sex, age, smoking status, marital status, physical activity, energy intake, and BMI. Abbreviations: LDL-c, Low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; VLDL, very low-density lipoprotein cholesterol; Triglyceride/HDL ratio, triglyceride/high density lipoprotein cholesterol ratio; Low density lipoprotein cholesterol/high density lipoprotein cholesterol; total cholesterol/HDL, total cholesterol/high density lipoprotein cholesterol; REF, reference.

Table 5. Linear regression analysis evaluating the associations between total urine resveratrol (independent factor) and liver status markers (dependent factor) in participants with MetS.

	Total Urine Resveratrol Metabolites (nmol/g Creatinine)			
	T1	**T2**	**T3**	***p*-Trend**
	(≤4.6)	**(>4.6 to 58.1)**	**(>58.1 to 2481.2)**	
n	**89**	**89**	**88**	
		β Coefficient (95% IC)	**β Coefficient (95% IC)**	
Liver markers				
Log ALT (U/L)				
Crude model	0 REF.	0.03 (−0.12, 0.18)	−0.10 (−0.25, 0.05)	0.074
Adjusted model	0 REF.	0.03 (−0.11, 0.18)	−0.12 (−0.27, 0.02)	0.028
Log AST (U/L)				
Crude model	0 REF.	0.003 (−0.10, 0.10)	−0.09 (−0.19, 0.01)	0.040
Adjusted model	0 REF.	−0.01 (−0.11, 0.09)	−0.12 (−0.22, −0.02)	0.011
Log GGT (U/L)				
Crude model	0 REF.	0.02 (−0.15, 0.20)	−0.23 (−0.41, −0.06)	0.002
Adjusted model	0 REF.	0.01 (−0.17, 0.19)	−0.24 (−0.42, −0.06)	0.002
HSI *				
Crude model	0 REF.	−0.28 (−1.74, 1.18)	−0.59 (−2.45, 1.27)	0.893
Adjusted model	0 REF.	0.14 (−1.35, 1.63)	0.11 (−1.37, 1.59)	0.948
FLI ¶				
Crude model	0 REF.	−0.97 (−5.46, 3.52)	−2.55 (−7.07, 1.98)	0.294
Adjusted model	0 REF.	−1.39 (−5.97, 3.18)	−2.54 (−7.10, 2.02)	0.346

Models were adjusted for sex, age, smoking status, marital status, physical activity, energy intake, and BMI. * Adjusted for all variables except for sex and BMI. ¶ Adjusted for all variables except for BMI. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HSI, hepatic steatosis index; FLI, fatty liver index; REF, reference.

2.3. Risk of Higher Liver Enzymes and Total Urine Resveratrol Metabolites

We tested the associations of total urine resveratrol metabolites and the risk of higher ALT (A), AST (B), and GGT (C) levels (Figure 1). Cubic splines analyses indicated that participants who had total urinary resveratrol concentration threshold had a lower odds ratio for liver enzymes above the ULN. Urinary resveratrol metabolites concentration threshold: ALT (169.2 to 1314.3 nmol/g creatinine), AST (559.9 to 893.8 nmol/g creatinine), and GGT (169.2 to 893.8 nmol/g creatinine).
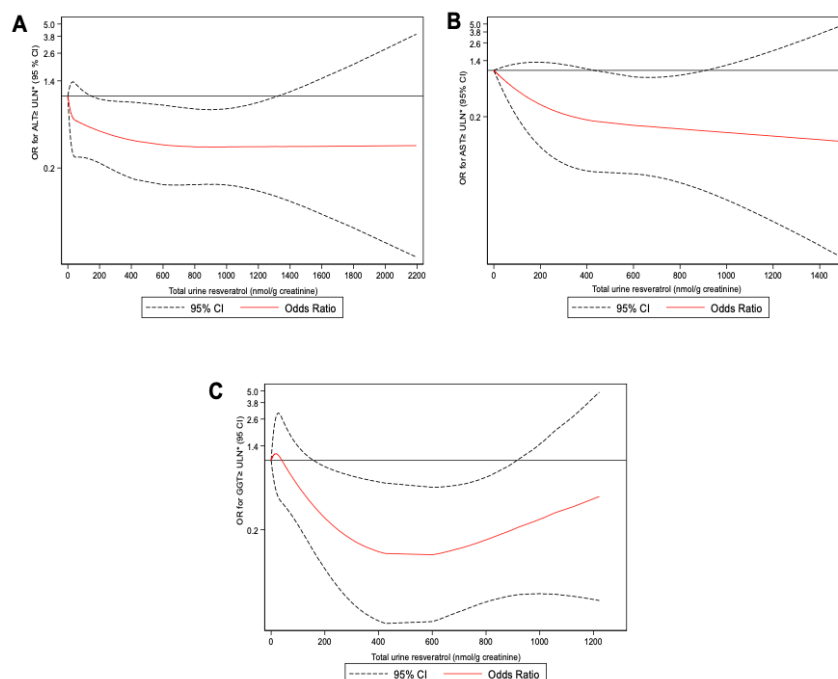
Figure 1. The odds ratio for liver enzymes levels above the upper limit of normal (ULN) for total urine resveratrol concentration in nmol/g creatinine. ULN range for ALT (men ≥ 30 UI/L, women ≥ 19 UI/L) (**A**), AST (men ≥ 37 UI/L, women ≥ 31 UI/L) (**B**), and GGT (men ≥ 60 UI/L, women ≥ 40 UI/L) (**C**). The smooth line represents the estimation of higher ALT, AST, and GGT levels when using zero as the reference value for total urine resveratrol metabolite (4 knots for ALT and GGT; 3 knots for AST) whereas the dashed lines indicate 95% CIs.

3. Discussion

In this research, higher urine concentrations of some resveratrol phase II metabolites (total sum of *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4′-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4′-*O*-glucuronide, and *trans*-resveratrol-3-*O*-sulfate) have associated with favorable lipid and liver markers in individuals diagnosed with MetS. Indeed, cubic spline models suggest that total urinary resveratrol excretion was associated with a lower risk of higher levels of liver enzymes related with increased risk of NAFLD (concentration threshold for ALT = 169.2 to 1314.3 nmol/g creatinine, AST = 559.9 to 893.8 nmol/g creatinine and GGT = 169.2 to 893.8 nmol/g creatinine), even after adjustment for potential factors. MetS components increase the risk of NAFLD development [2–4]. In general, our population had an abnormal metabolic profile as characteristic of MetS where women showed higher cholesterol and LDL-c levels compared to men. Meanwhile, men exhibited higher levels of ALT, AST, and FLI. Epidemiological studies evidenced that age and sex affect NAFLD prevalence [3]. Ageing involves changes in sex hormones levels, fat redistribution that increases the risk of CVD and NAFLD, especially in post-menopausal women [30,31]. Healthy dietary patterns, such MedDiet, include foods that not only might improve weight modulation, but also have several bioactive compounds like (poly)phenols with anti-inflammatory and antioxidant properties, which show beneficial metabolic effects [9,10,32,33]. Some molecules, such as anthocyanidins and resveratrol, might involve in the metabolic process involved in NAFLD [15,29]. Scientific evidence suggested that resveratrol is a multi-targeted compound for chronic

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diseases [21]. However, variations in the study design, small samples sizes, diverse analytical methods, and other factors trigger heterogeneous conclusions. Consequently, results should be interpreted cautiously [24,29,33,34]. Interestingly, our findings showed that individuals in the highest tertile of total urinary resveratrol metabolites had lower levels of triglycerides, VLDL-c and total cholesterol/HDL ratio compared with those in the lowest tertile. Previously, the PREDIMED study (PREvención con DIeta MEDiterránea) evaluated the association of cardiovascular risk factors and total urinary resveratrol metabolites [35]. Authors demonstrated that increased urinary resveratrol excretions were associated with higher HDL-c, lower triglycerides concentrations and decreased heart rate, but did not found associations with blood pressure [35]. While in our results, no differences were found in the HDL-c concentrations. The discrepancies between our results related to the lipid metabolism could be partly explained for the T2DM status attributable to the synergetic effects of anti-diabetic drug and resveratrol as well as lipid-lowering medication [28]. It is important to mention that incidence of T2DM in our population was lower compared to Zamora et al., reported [35]. Moreover, when we adjusted the regression models considering lipid-powering and anti-diabetic treatment, our results did not change (data not shown). Another difference between both studies is that we quantified slightly different resveratrol metabolites. We quantified *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4′-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4′-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate while Zamora-Ros et al. quantified (*trans*-/*cis*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4′-*O*-glucuronide, *trans*-/*cis* resveratrol-4′-*O*-sulfate, *trans*-/*cis* resveratrol-3-*O*-sulfate). Furthermore, we used authentic glucuronide and sulfated standards to quantify each metabolite. In contrast, Zamora-Ros et al. used the resveratrol aglycone to quantify all glucuronide and sulfated metabolites, which can lead to errors in the quantification of glucuronide and sulfated metabolites [36]. The lack of commercially available glucuronide and sulfate resveratrol standards is still an issue that hampers advancements in the quantification of total resveratrol metabolite. The lipophilic nature of resveratrol could facilitate the entry into the surface of albumin and lipoprotein, and these properties could confer benefits on lipid profile, avoiding the oxidation of LDL [28,37,38]. A study found that resveratrol metabolites, including *trans*-/*cis*-resveratrol-3-*O*-glucuronide, and *cis*-resveratrol-3-*O*-glucoside, as well as free *trans*-resveratrol, were incorporated into the LDL of human participants after intake of moderate red wine, which could suggest the cardioprotective role of resveratrol on atherogenic markers and oxidative stress [37]. In line with our findings, a meta-analysis indicated that more prolonged resveratrol supplementation (≥6 months) with doses ranged from 8.1 to 3000 mg/d might improve triglyceride levels in subjects with T2DM [28]. However, in a prospective cohort study, Semba et al. did not find significant differences in lipid profile and inflammatory cytokines across groups of total urinary resveratrol [39]. It is essential to highlight that our participants had MetS, which are closely related to NAFLD due to deregulation of the de novo lipogenesis (DNL), insulin resistance, and hepatic triglyceride accumulation [5,6]. Thus, these findings suggested that resveratrol could improve liver parameters. Several mechanisms of resveratrol action on lipid metabolism include the activation of AMP-activated kinase (AMPK), which inhibit sterol regulatory element-binding protein 1 (SREBP-1) activity, which plays a crucial role in the DNL [28,40]. Moreover, the regulation of hepatic enzyme 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) related to cholesterol synthesis [38], and the overexpression of the paraoxonase 1 (PON 1) that it has shown cardioprotective effects [41]. In fact, disrupted SREBP-1 levels, increased HMG-CoA expression and decreased PON 1 activity have evidenced in NAFLD promoting a dysregulation of lipid metabolism [28,38,40,42]. This environment stimulates the accumulation of lipids into hepatocytes (liver steatosis) increasing risk to develop NAFLD [7,43]. In the current study, there were no statistically significant differences in glucose metabolism markers among tertiles of total urine resveratrol metabolite, but so far the effect of resveratrol on glucose metabolism is unclear [44]. A randomized controlled trial did not show significant effects in HOMA-IR and fasting glucose levels after four weeks of supplementation with 150 mg of *trans*-resveratrol in subjects who were overweight [45]. In contrast, a recent meta-analysis has shown that resveratrol supplementation (≥100 mg/d) might reduce levels of insulin and glucose in individuals diagnosed with T2DM [27]. Regarding liver parameters, 46% of the participants had

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ALT values above ULN, and higher total urinary resveratrol metabolites were significantly associated with lower AST and GGT levels. We also observed in the cubic spline analyses that total urinary resveratrol metabolite (concentration threshold) reduced the probability of having higher liver transaminases (ALT, AST and GGT). In this respect, clinical trials studies focus on resveratrol effects on NAFLD individuals are scarce with ambiguous results [46,47]. For instance, Chen et al. showed that resveratrol supplementation (600 mg for 3 months) could decrease levels of liver transaminases, LDL-c, total cholesterol and HOMA-IR in, but did not found a significant reduction in liver steatosis. However, another study indicated that lifestyle changes focused on a healthy diet and physical activity in addition to 500 mg/d (12 weeks) of resveratrol supplementation only had beneficial effects on improvements in ALT levels and hepatic steatosis [48]. In contrast, a study showed that insulin resistance markers and hepatic steatosis remained unchanged after resveratrol supplementation [49]. Contrary to our study, Cachay et al. only included men in their study design. In fact, it has suggested that stilbene glucuronidation is more efficient in women compared to men [50]. Moreover, there are significant differences in the doses. Cachay et al. used up to 20 times higher the amount compared to other research groups. In this sense, our contrasting results can be explained by the fact that chronic higher resveratrol doses might promote saturation in absorption sites [51]. Our findings suggested that inter-individual heterogeneities might play a key role in the effectiveness of resveratrol metabolites in individuals with MetS who are overweight or obese. However, it should be noted that the studies, as mentioned above, are different in study design, populations, and several other aspects that could potentially affect results and the interpretation of their conclusions.

There is a lack of epidemiological research in evaluating the effects of resveratrol from dietary consumption and health outcomes [21,27,28,39]. On the other hand, the majority of clinical trials assessed the effects of resveratrol supplementation using diverse resveratrol dosage and frequency of intake and heterogeneous treatment lengths. Therefore, it is difficult to interpret results and establish an effective dose and treatment, especially for the use of higher amounts, which is not applicable in a normal dietary context. Resveratrol is mainly found in wine, grapes, and grape juice [18,52,53]. In our data, wine consumption was correlated with urinary resveratrol metabolites (data not shown). However, resveratrol content can vary in the same type of fruit, climate, and grape variety for wine [53,54]. The bioavailability of resveratrol is poor, resulting from low water solubility (<0.05 mg/) that can vary according to the matrix (wine, grapes, supplements, others) [20,24,54]. Rotches-Ribalta et al. evaluated resveratrol metabolites profiles after a moderate intake of red wine and grape extract tablets in healthy men [20]. Investigators found differences in the quantification of some resveratrol metabolites due to the different resveratrol composition of both sources [20]. A large number of human and animal studies suggested that bioactive phytochemicals have therapeutic effects on chronic diseases, but several factors may affect their biological response [19,25]. The main determinants of inter-individual variation could be attributed to the gut microbiota, sex, age, lifestyle, genetics, and others [25]. In this line, it seems essential to consider that resveratrol metabolites could have beneficial effects on specific population groups, where inter-individual variances in their metabolism could confer to these discrepancies [21,25,27,28,39]. Consequently, conflicting views about the effect on the metabolic profile of resveratrol in a supplementation or food form are still unclear.

Current recommendations for NAFLD prevention, treatment, and follow-up encourage lifestyle modifications to focus on habitual physical activity and healthy dietary patterns. From a dietary point of view, it is a challenge to promote healthy diets focused on foods with high content in bioactive compounds. The MedDiet could be a preferable option to be considered since its dietary components are rich in antioxidants, which are pivotal factors for the prevention and management of NAFLD [9–15]. In this context, well-design epidemiological and clinical trial studies to investigate the effects of dietary resveratrol on health outcomes are crucial.

The strength of this study is the large sample size of patients with detailed clinical and biochemical data. Moreover, the use of metabolomics has been considered a reliable and innovative technique for food science and precision nutrition studies [8]. In the present study, we performed an analytical method to accurately identify and quantify resveratrol metabolites using authentic

standards. However, our study has some limitations. First, for total urine analyses, not all phase II metabolites were included, and we did not use glucosides and gut microbial metabolites, which can lead to underestimation of total resveratrol metabolite levels and, therefore, influence our conclusions. Hence, data are not representative of total resveratrol intake, and we included the main resveratrol phase II metabolites reported in human studies [20,21,53] and also considering the limited availability of authentic standards. Second, liver biopsy was not performed for diagnosing NAFLD participants. In this sense, non-invasive markers were acceptable to identify patients with metabolic features at risk of developing NAFLD [4,55] Finally, this study has a cross-sectional design, and the findings cannot infer causality. Likewise, results cannot be generalized to other ethnic or age groups, because the participants were elderly diagnosed with MetS at high CVD risk. However, type I and II errors cannot be discarded, despite that, the results are plausible and with clinical relevance.

4. Materials and Methods

4.1. Study Population

Participants were volunteers from the PREDIMED-Plus study, a parallel-group multi-center randomized trial (https://www.predimedplus.com/). Details of the study design have been previously described [56]. In brief, PREDIMED-Plus study was designed to investigate the effects of an energy-reduced Mediterranean diet and a weight-loss intervention by the promotion of physical activity and behavioral support on cardiovascular endpoints [57]. Individuals were men and women (65 to 75 years) who were overweight or obese and met at least three components of the MetS [58]. The study excluded participants with excessive alcohol consumption or addiction, several medical conditions (active cancer or history of malignancy, history of previous CVD, cirrhosis or liver injury, cytotoxic agents, therapy with immunosuppressive drugs, or treatment with systemic corticosteroids [56]. All participants gave their informed consent to participate in the study. This clinical trial was conducted following the Declaration of Helsinki, and the protocol was approved by institutional ethics committees of all participant centers (http://www.isrctn.com/ISRCTN89898870). This study is a cross-sectional study using baseline database from the Navarra-Nutrition node. A total of 266 participants with feasible data in the form of spot urine specimen were included in the present study.

4.2. Sociodemographic, Clinical, Anthropometric, and Body Composition Variables

Sociodemographic characteristics, lifestyle data, and medical history were collected during the baseline interview according to the study protocol [56]. Smoking habit was classified into never, former, and current smoker. Diabetes was defined according to the criteria of the American Diabetes Association guidelines [59]. Anthropometric variables were measured by trained dietitians using standardized procedures and calibrated equipment [56]. Height (in centimeters) and weight (in kilograms) were measured to calculate body mass index (BMI) (kg/m^2). Visceral fat mass was estimated using the dual-energy X-ray absorptiometry (Lunar iDXA ™, software version 6.0, Madison, WI, USA) performed by trained study staff. We used a validated Registre Gironi del Cor (REGICOR) questionnaire to assess physical activity (Metabolic Equivalent of Task (MET)-minute/week, as described in detail elsewhere [60–62].

4.3. Dietary Record

A validated 143-item semi-quantitative food frequency questionnaire was administered in a face to face interviews by a trained nutritionist to explore dietary intake over the previous 12 months [63]. Furthermore, adherence to MedDiet was assessed by a 17-point score questionnaire, which is a version of the 14-point score performed in the PREDIMED study [56,64,65]. The 17-point score questionnaire includes additional questions to the 14-point score and more restrictive cut-offs for some caloric-dense foods [56,65].

4.4. Urine and Plasma Collection, and Biochemical Determinations

The first spot urine was taken in the morning, and blood samples were obtained after 12 h overnight fasting. Biological specimens were stored frozen at a −80 °C according to approved protocols by trained technicians [56]. Biochemical analyses including glucose, hemoglobin A1c (HbA1c), triglyceride, HDL-c, total cholesterol, ALT, and AST were performed on fasting plasma by using specific kits according to manufacturer's protocols [56]. The insulin was measured using specific ELISA kits in a Triturus autoanalyzer (Grifols, Barcelona, Spain). The Friedewald formula was used to calculate LDL-c and the VLDL-c [66].

4.5. Urine Resveratrol Metabolites Measurements

Standards of *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4'-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4'-*O*-glucuronide, and *trans*-resveratrol-3-*O*-sulfate were obtained from Toronto Research Chemicals (Toronto, ON, Canada). The resveratrol metabolites were extracted and quantified using a modified method developed by Feliciano et al. (2016) [67]. The analytical method was validated according to the Food and Drug Administration (FDA) guidelines. Briefly, 600 μL of diluted urine samples (urine:water, 1:10) were thawed on ice and centrifuged at 15,000× g for 15 min at 4 °C. Then the supernatant (350 μL) was transferred to a microtube and acidified with 4% phosphoric acid. The mixture (600 μL) was loaded onto Oasis 96-well reversed-phase hydrophilic-lipophilic balanced (HLB) sorbent μ-SPE plates (Waters, Eschborn, Germany) and eluded with 60 μL of methanol after washing. Isotope labelled standards (±)-Catechin-2,3,4-13C3 (0.54 mg/mL, Sigma-Aldrich, Steinheim, Germany) and ferulic acid-1,2,3-13C3 (0.99 mg/mL, Sigma-Aldrich, Steinheim, Germany) were spiked in samples before μ-SPE to indicate the recovery rate. Taxifolin (0.25 mg/mL, Sigma-Aldrich, Steinheim, Germany) were used as internal standard. The identification and quantification of resveratrol metabolites was performed on a Shimadzu Triple Quadrupole Mass Spectrometer (LCMS8060, SHIMADZU, Kyoto, Japan) through an electro-spray interface (ESI) source. Eluded samples (5 μL) were injected through a Raptor Biphenyl column 2.1 × 50 mm, 1.8 μm (Restek, Bellefonte, PA, USA) with a compatible Raptor Biphenyl Guard Cartridges 5 × 2.1 mm (Restek, Bellefonte, PA, USA) in the UPLC system. The mobile phases consisted of solvent A: water (HPLC grade, Sigma-Aldrich, Steinheim, Germany) with 0.1% formic acid (LC-MS grade, Thermo Fisher Scientific, Loughborough, UK), and solvent B: acetonitrile (HPLC grade, Sigma-Aldrich, Steinheim, Germany) with 0.1% formic acid. A fourteen-minute gradient joined by a two minutes equilibration was applied to the run under a flow rate of 0.5 mL/min at 30 °C. The gradient was as follows (t(min), %B): (0, 1), (1, 1), (4, 12), (8, 12) (8.1, 15), (11, 15), (11.5, 30), (12, 99), (14, 99), (14.1, 1), (16, 1). The MS/MS parameters and transitions of the target compounds were obtained in optimization run. The resveratrol metabolites in samples were identified by comparing retention times with standards in corresponding to the multiple reaction monitoring (MRM) transitions and quantified by calibration curves made from standard mixes. One pair of isomers *cis*-resveratrol-3-*O*-glucuronide and *cis*-resveratrol-4'-*O*-glucuronide were quantified together as they appear in the same retention time. The identification of each metabolite was based on retention time of its corresponding pure standard following the same conditions and reference ion ratios based on the MS optimizations. Urinary resveratrol metabolites were normalized for urine creatinine concentrations.

4.6. Glucose Homeostasis and Liver Markers Measurements

Glucose homeostasis markers, such as insulin resistance and insulin sensitivity, were calculated using the homeostasis model assessment for insulin resistance (HOMA-IR) [68], as well as the homeostasis model assessment for β-cell function (HOMA-%B) [69], the fasting glucose insulin ratio (FGIR) [70], and the fasting insulin resistance index (FIRI) [70]. Moreover, non-invasive liver markers such as the hepatic steatosis index (HSI) [55,71] and the fatty liver index (FLI) [72] were also determined estimated considering clinical, biochemical and anthropometric data. Formulas followed for all these determinations were as follows:

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$$HOMA - IR = Insulin(\text{mU/L}) * glucose \text{ (mmol/L)}/22.5$$

$$HOMA - \%B = Insulin(\text{mU/L})/glucose \text{ (mmol/L)} - 3.5$$

$$FGIR = glucose \text{ (mmol/L)}/Insulin(\text{mU/L})$$

$$FIRI = Insulin(\text{mU/L}) * glucose \text{ (mmol/L)}/25$$

$$\text{HSI} = 8 \times \text{ALT/AST ratio} + \text{BMI} (+2, \text{if diabetes}; +2, \text{if female})$$

$$\text{FLI} = \frac{e^{0.953 \times \log(\text{triglycerides}) + 0.139 \times \text{BMI} + 0.718 \times \log(\text{GGT}) + 0.053 \times \text{waist circumference} - 15.745}}{1 + e^{0.953 \times \log(\text{triglycerides}) + 0.139 \times \text{BMI} + 0.718 \times \log(\text{GGT}) + 0.053 \times \text{waist circumference} - 15.745}} \times 100$$

4.7. Statistical Analysis

Descriptive statistics were shown as means and standard deviation (SD) for continuous variables, and *n* (%) for categorical variables. A chi-squared test for categorical variables and Student's *t*-test were used to compare baseline characteristics of participants by sex. Participants were categorized according to tertiles of some urinary resveratrol phase II metabolites excretion (T1 = ≤4.6 nmol/g; T2 = >4.6 to 58.1 nmol/g; T3 = >58.1 to 2481.3 nmol/g creatinine). Unadjusted and adjusted linear regression models were used to analyze the relationship between total urine resveratrol metabolite and cardiometabolic profile and NAFLD risk markers.

The normality of the residuals was tested in order to assess the validity of the regression models. Variables such as triglycerides, Triglyceride/HDL ratio, ALT, AST, and GGT were markedly skewed and were log-transformed. Linear regression analysis was adjusted for sex (except for covariates that include sex), age, smoking status (never, former, current), marital status (single, married, widow, divorced, separated, others), physical activity (MET-min/week), energy intake (kcal/d), and BMI (except for covariates that include BMI). Tests of linear trend were performed assigning the median value of each tertile of total resveratrol urine metabolite and then using it as a continuous variable.

We applied flexible cubic spline models to evaluate the association of total urinary resveratrol metabolites (continuous variable) with liver enzymes above the ULN. For ALT (men ≥ 30 UI/L, women ≥ 19 UI/L) [73], AST (men ≥ 37 UI/L, women ≥ 31 UI/L) [74], and GGT (men ≥ 60 UI/L, women ≥ 40 UI/L) [75]. Models were adjusted by all variables previously mentioned except for sex and included total sleeping hours (h/d). In the cubic spline analysis for total urinary resveratrol metabolites, we used 0 as a reference, with 4 knots (ALT and GGT) and 3 knots (AST). Statistical tests were two-tailed, and the significance level was *p* < 0.05. All statistical analyses were conducted with STATA version 16.0, StataCorp LP, College Station, TX, USA.

5. Conclusions

Current data showed that high urinary levels of some resveratrol phase II metabolites were associated with better blood lipid profile and liver enzymes in individuals diagnosed with MetS. Moreover, urinary resveratrol concentration threshold is associated with a reduced risk of higher liver enzymes. These results suggested that some resveratrol metabolites might have associated with benefits on risk factors linked to NAFLD development. Further studies are warranted to elucidate the impact and effectiveness of resveratrol in liver outcomes in individuals with MetS.

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Sample Availability: Samples of the compounds are available from the authors.

V.   DISCUSSION

1. **Justification of the study**

Nowadays, NAFLD has become one of the most important features of chronic liver diseases being a potential contributor to the clinical burden due to chronic hepatic complications, liver-morbidity and mortality [1,5,7]. The incidence of NAFLD is estimated to be higher in men compared to women in adulthood, but these trends change in the elderly population [1,53]. The NAFLD pathogenic mechanism based on the two hits hypothesis includes the lipid accumulation in the hepatocytes in the first hit, which increase the susceptibility of liver damage caused by oxidative stress and the activation of proinflammatory pathways in the second hit [8,32]. Then, hepatocytes may lead to a constellation of lesions (i.e., inflammation, ballooning and fibrosis), promoting the evolution from simple steatosis to NASH [8]. In this context, central obesity, T2DM and MetS could be the driving risk factors toward a more progressive and severity disease phenotype [2,5]. Disrupted VAT induced an up-regulation of proinflammatory mediators and insulin resistance driven towards liver failure [58,212]. Generally, abdominal obesity is measured by waist circumference. However, this anthropometric measure may not accurately reflect VAT in all subjects [213]. In the current study, body composition was measured by DXA that is considered the gold standard [214].

Unhealthy dietary patterns and sedentarism are associated with unfavourable impact on metabolic profile leading to liver damage [1,2]. Current expert recommendations suggest that lifestyle modifications, emphasizing a healthy diet and improvements in physical activity could be essential in the primary prevention and management of NAFLD [1,2,110,111].

The MedDiet is characterized by higher and balanced consumption of healthy foods rich in bioactive compounds that are associated with several benefits on body weight and health outcomes [112–114,116–118]. Bodyweight reductions could reverse liver disease by the resolution and improvements in histological features of NAFLD [1]. In this sense, the MedDiet has been proposed as a suitable dietary pattern for this goal [110,111,124,125]. For this purpose, we applied an FFQ based on 143 items, which was previously validated in the Spanish population. Also, adherence to the MedDiet was evaluated by the 17-item score, which is a

modified scale from the 14-point score (MEDAS)[195] and the Mediterranean Diet score formulated for Trichopoulou et al. [215] These MedDiet scores have been widely used in previous studies [196,209,210,215]. Furthermore, we used target metabolomics to analyze the food metabolome, using authentic standards of resveratrol metabolites. This point should be particularly highlighted, considering that metabolomics has been a powerful approach for the quantitative analysis of polyphenols [216,217].

Liver biopsy is considered the reference for NAFLD diagnosis [82], but this procedure underlies several complications, and it is not considered as a suitable tool in the primary health setting and epidemiological-large studies [218]. Therefore, this research includes several non-invasive liver markers that have been shown good accuracy to estimate NAFLD [67,68,92,94,219]. In the present study, we used the TyG index, which is considered as a reliable alternative indicator for insulin resistance [65,220]. Also, this marker has exhibited a higher sensitivity in detecting NAFLD [221].

In this thesis, we focused on the influence of sex-differences on metabolic phenotyping and lifestyle factors on diet differences such as MedDiet adherence, food preferences, different bioactive compounds, physical activity, and their relationship with the onset of NAFLD in individuals diagnosed with MetS. The importance to investigate these interrelationships may help to recognize individuals at high risk of NAFLD development as well as to improve in the prevention and management of this disease.

2. Different sex signatures in VAT and insulin resistance associated with NAFLD risk factors

MetS comprises several cardiometabolic manifestations, which is commonly present in individuals who were obese [222,223]. Several scientific sherds of evidence suggested that MetS is strongly associated with NAFLD development and disease severity [223–225]. However, the biological and pathophysiological mechanism for this link remains uncertain.

Considering the limitations of current NAFLD diagnosis, clinical investigations have evaluated novel non-invasive liver markers related to the pathological mechanisms involved in NAFLD [219,226]. Some of these liver indicators/surrogates are the HSI, FLI, and TyG index, which have shown good accuracy for recognizing NAFLD [92,94,219,226–228]. In this context, we explored potential NAFLD risk factors in a sub-group analysis considering men and women with lower alcohol intake (Chapter 1). Our results showed that women had a higher risk to have increased HSI values compared to men. NAFLD is more prevalent in men than women in young adults, but these rates changed in the elderly population [1,53,229,230].

It is important to mention that age-related changes have also been involved in several pathological alterations in lipid and glucose metabolism, and liver functions [230]. Frith et al. showed that individuals diagnosed with NAFLD, older participants presented more metabolic risk factors and greater fibrosis than younger subjects [231]. Moreover, a retrospective study case-control indicated that the prevalence of liver steatosis was higher in older compared to middle-aged participants, which increased from 2.7% to 28% in older women [231]. In line with our data, some studies revealed that the prevalence of NAFLD was associated with menopausal status and estrogen medication in females [232,233] and their prevalence increased in older females [234]. Likewise, Zhou et al. showed that NAFL rates were higher in men than women under the age of 50 years, but this prevalence significantly changed in participants over the age of 50 years [234]. These results may be explained in part by the decrease in estradiol concentration observed in menopause, which promotes a reduction in the capacity of the FFA's oxidation leading to hepatic lipogenesis and steatosis [53]. At the same time, choline biosynthesis is also affected, inhibiting the delivery of triglycerides from the liver [53]. These aspects are relevant during the menopausal period where sexual hormones could play a crucial role in NAFLD pathogenesis [53,230].

Interestingly, our findings indicated that individuals with increased VAT, as measured by dual x-ray absorptiometry, showed worse lipid profile, glucose metabolism and hepatic

markers (Chapter 2). According to our results, a study reported that higher levels of ALT were dose-dependently associated with large amounts of VAT in participants with NAFLD [235]. Lee and investigators showed that VAT could be an independent risk factor for hepatic steatosis [236]. In the same way, another study revealed that increased VAT was independently associated with NASH or significant fibrosis in subjects diagnosed with NAFLD [237]. It is must be pointed out that 30% of hepatic FFA delivery are originated from VAT lipolysis in subjects with higher amounts of VAT [12]. Furthermore, the VAT might seem to be a crucial factor for the development of atherogenic dyslipidaemia characterized by abnormal HDL-c, triglycerides, and non-HDL-cholesterol levels [238]. On the other hand, Neeland et al. evaluated the effects of VAT on hepatic gluconeogenesis pathways in subjects who were obese without T2DM [239]. Investigators suggested that greater amount of VAT, overstimulating hepatic gluconeogenesis [239]. This state, due to the increased delivery of glycerol from mesenteric triglyceride turnover into the portal circulation and liver, increasing the risk of T2DM [239].

Plausible mechanisms involved included that expanded and disrupted endocrine functions of adipose tissue may lead to an adiposopathy response [212]. This pathological state results in large amounts of ectopic fat (liver, heart, pancreas, and others), abnormal redistribution of VAT, activation of several inflammatory processes, adipokine dysregulation and insulin resistance [212]. In fact, VAT is the primary source of FFAs and proinflammatory cytokines, promoting hepatic steatosis that supports this association [58,212].

On that basis, our data reinforce that enlarge VAT has pro-atherogenic effects on lipid profile and a negative impact on NAFLD features in older individuals.

The TyG index is a single and reliable marker, which could predict T2DM [65,220]. This fact has to be taking into consideration because T2DM is a strong predictor of advanced fibrosis and mortality in patients diagnosed with NAFLD [240]. Moreover, the TyG index has shown to be a useful predictor of hepatic steatosis and NASH [221]. Our data revealed that patients with TyG values above 9.1 presented significantly higher levels of HOMA-IR, ALT, GGT, FGF-21, FLI and HSI (Chapter 2).

In line with our results, Zhang et al. found that TyG values above 8.5 had high accuracy (AUC: 0.782; 95%CI: 0.773–0.790), within identifying individuals with NAFLD [227]. The ability of the TyG index as a predictor of NAFLD has also been found in a study, where the cut-off level of 8.52 (AUC: 0.76; 95%CI: 0.74–0.77) compared to triglycerides, ALT and fasting plasma glucose [68]. Recently, a study reported that the TyG index presented the highest AUC for diagnosing MetS compared to other markers [66]. In another study, authors found significant associations between the TyG index and metabolic risk factors. Also, it showed as an independent predictor of CHD [241]. Likewise, Zhou et al. reported that the TyG index was associated with a higher risk of stroke recurrence, all-cause mortality, and worsening prognosis in subjects diagnosed with ischemic stroke [242]. In this sense, our results point to the usefulness of the TyG index as an easy and proxy indicator for NAFLD risk factors.

Remarkably, we found that the TyG index was able to discriminate VAT in women, but it was weak for men. To the best of our knowledge, this is the first study to evaluate the TyG index as a predictor of VAT in participants with MetS. These findings offer overwhelming evidence that sex difference in body fat distribution could contribute to the disparity in insulin sensitivity [243,244] and cardiovascular risk factors [245]. For instance, Geer et al. observed that enlarger amounts of VAT could be related to higher insulin resistance in women than men because of the lack of estrogen effects [244]. In contrast, Ferrara and colleagues found that men with obesity were more insulin resistance despite body fat distribution compared to women in older participants [243]. However, it is important to consider that Ferrara et al. included a small sample might give to this conflicting result. Moreover, a cross-sectional study with 2,983 obese individuals who observed that VAT was significantly associated with increased cardiometabolic risk in women (OR: 5.77; 95% IC: 3.02, 11.01) compared to men (OR: 1.42; 95% IC: 0.84, 2.41) [245].

Our results could be explained by the sex-based differences in body fat distribution [52,212]. Generally, females have a higher percentage of total body and gluteal-femoral fat mass, whereas males presented more VAT [52]. Nevertheless, this body fat distribution pattern usually changes in ageing [1,53]. Moreover, it has been argued that differences in the distribution of adiposity are linked to metabolic disorders [246]. In fact, the gluteal-femoral fat distribution could have protective effects on glucose and lipid profile, decreasing risk to develop CVD [52,212]. In menopausal status, decreased levels of estrogens have also been involved in the re-distribution of fat storage towards a more accumulation of fat mass in the visceral region [1,53]. Another mechanism implicated could be that postmenopausal women with MetS exhibited decreased LPL activity leading to a limited capacity for subcutaneous adipocyte lipid storage and increased VAT deposition [247]. Also, females might display large amounts of FFA's delivery derived from VAT lipolysis than males [12].

Finally, our results should point out that VAT and altered insulin signalling play a pivotal role in metabolic features linked to NAFLD, considering that VAT and sex could be a stronger predictor of VAT FFA's delivery to the liver [12]. Besides, the TyG index could be a useful non-invasive marker to identify abnormal VAT in women. This finding could constitute a novel tool for healthcare professionals to screening individuals with a high risk of NAFLD. However, further research is required in order to elucidate the plausible mechanism for these associations.

3. Lifestyle modification on the NAFLD risk factors

Lifestyle interventions, including a healthy diet and physical activity are essential factors to be considered in the prevention and treatment of NAFLD [1,103]. Weight loss promotes a balance in metabolic profile, resulting in a lower risk to develop CVD, T2DM but also can improve NAFLD progression [1]. Bodyweight reduction at least of 5% has shown improvements in hepatic steatosis, while ≥10% weight loss has associated with resolution of NASH features such as portal inflammation and fibrosis [1].

Excessive caloric intake leads to obesity and MetS, increasing the risk to develop NAFLD [105,109]. A growing body of evidence proved that MedDiet is an effective dietary strategy in reducing the risk of non-communicable diseases such as CVD and NAFLD [1,2,5,110–112]. The protective role of MedDiet on metabolic disorders can be attributed to synergy between the lower glycaemic content, nutrients, bioactive compounds with metabolic-regulating properties as well as antioxidant and anti-inflammatory effects [109–112,134,248–251]. An study evidenced that a higher dietary total antioxidant capacity was inversely associated with insulin resistance [248]. Also, increased HOMA-IR values showed a positive correlation with the glycaemic index in individuals with NAFLD [248]. In the PREDIMED-Plus also it has shown that the improvement in the carbohydrate quality index was strongly associated with reductions on CVD risk markers after 6- and 12-months [249].

Fruits and vegetables are one of the most abundant dietary components in the MedDiet. In this line, a study revealed that fibre from fruits is closely related to better liver health in participants with MetS [134]. Also, it has indicated that a higher insoluble fiber intake (≥7.5 g/day) could improvement liver fibrosis [251]. Furthermore, the intake of 800 g/day of fruit and vegetables has been associated with a reduced risk of CVD and all-cause mortality [250]. In the MedDiet, the consumption of meat and dairy products is lower because of the highest content of detrimental components such as saturated fatty acids [112]. It has suggested the connection between the intake of red meat and metabolic diseases [128,252–254]. Higher consumption of red meat (rich in saturated fat) was positively associated with abdominal obesity, hypertriglyceridaemia and MetS opposed to the intake of white meat [252]. Likewise, Zelber-Zagi et al. reported that increased consumption of red and processed meat was associated with insulin resistance and NAFLD [253]. Also, investigators from the PREDIMED-plus showed that dietary fat intake was associated with higher odds for risk of hyperglycaemia [255]. At the same time, dietary amino acids could be influenced by glucose and liver metabolism in individuals with NAFLD [254]. In this sense, our study was to widen further current knowledge of the relationship between adherence to MedDiet and dietary components in NAFLD risk factors.

Dietary scores have been recognized as a useful tool with practical applications, concerning the degree of adherence and their benefits on health outcomes [256]. The Mediterranean dietary score used in our study is in line with a variation of that used by Trichopoulou et al. [209,210]. Several studies have applied this score and have reported inverse associations between higher adherence to MedDiet and overall mortality, cardiac mortality and T2DM in different populations [209,210,215].

The most striking result to emerge from our study is that greater adherence to the MedDiet was inversely associated with HSI values, suggesting a lower risk for the development of NAFLD. Our data lend support to previous findings in many clinical and epidemiological studies [148,149,257]. In a cross-sectional study, Trovato et al. indicated that increased odds of NAFLD was associated with higher BMI, Western dietary patterns and lower adherence to the MedDiet [148]. Furthermore, in the Mediterranean Dietary Intervention study in Non-alcoholic fatty liver disease (MEDINA), individuals in the MedDiet group presented significant reductions in hepatic steatosis and better insulin sensitivity independently of weight loss compared to the low-fat diet (LFD) group [149]. Similarly, Moosavian et al. showed that MedDiet might improve anthropometric variables, lipid profile, glycemic indices, liver enzymes (ALT), and non-invasive liver scores on individuals with NAFLD [258].

Inflammatory state and oxidative stress have been common features in NAFLD and many metabolic diseases [259,260]. Individuals with MetS who were obese presented a proinflammatory state, leading to greater amounts of adipose tissue that releases increased levels of cytokines [58,260]. The connection with NAFLD is due to the exacerbated inflammatory response trigger to oxidative stress that activates several signal networks, causing hepatic apoptosis and liver damage [5,6,78,260]. Unhealthy dietary patterns characterized by a higher intake of refined starches, sugar, saturated and trans-fatty acids, sugar-sweetened beverages, and ultra-processed foods may promote the activation of the innate immune system and oxidative stress [261]. In our research, an increased level of cholesterol, triglycerides and large amounts of body fat regions was observed in participants with higher

HSI values. This fact it is interesting because in the PREDIMED-Plus cohort, a higher adherence to the MedDiet exhibited marginal associations with lower prevalence of individual and clustered cardiovascular risk factors (hypertension, obesity, T2DM, and dyslipidaemia) [257]. Therefore, the MedDiet has suggested as an outstanding dietary treatment against chronic low-grade inflammation-related diseases [111,112,261,262].

These benefits could be attributed to the higher consumption of omega-3 fatty acid from fish and plants, a lower intake of Omega-6: Omega-3 ratio (2:1-1:1) [261], and bioactive compounds [124] [126]. Estruch et al. revealed that MedDiet had a positive effect on inflammatory cytokines (IL-6, soluble intercellular adhesion molecule-1 and C-reactive protein) and decreased endothelial functions [112]. Also, the MedDiet could reduce tumour necrosis factor receptor (TNFR) concentrations in older patients at high cardiovascular risk [262]. In the ATTICA study, investigators revealed that total antioxidant capacity (measured by immune-diagnostic assay) was positively associated with higher MedDiet adherence and lower levels of oxLDL [111]. It should be noted that subjects diagnosed with NAFLD display pathogenic profile, which increased risk of all-cause events and CVD mortality [76].

Nowadays, there has been growing interest in legume consumption and their impact on health [263]. Despite this interest, there is a lack of studies in NAFLD [264]. On this basis, this study sheds new light on the association between legume consumption and NAFLD in individuals with MetS. The most marked observation to emerge from our study is that a higher intake of legumes (21 g/d) was associated with lower odds to be in the highest tertile of HSI. Our findings appear to be well substantiated by previous clinical studies that evaluated the influence of legume intake on metabolic disorders [263,265–268], bodyweight control [269], and NAFLD [264]. A study demonstrated an inverse dose-response association between the consumption of legumes and atherogenic profile [265]. Another similar article also reported that higher legume intake showed a decreased risk of 10% in CVD and CHD (relative risk: 0.90; 95 %CI: 0.84, 0.97) compared to lowest consumption of legumes [266].

Hermsdorff et al. showed that legume intake within a hypocaloric regimen could reduce body weight, LDL-c, total cholesterol and C reactive protein in subjects who were obese [267]. Furthermore, in the PREDIMED study found that greater legume consumption (28 g/d) was associated with a lower risk for T2DM in patients at high risk for CVD (hazard ratio: 0.65; 95% CI: 0.43 to 0.96; P-trend= 0.04). Also, researches indicated that replacing of a half a serving/d of legume (30 g) for other foods such as eggs, wholemeal and white bread, rice or baked potato, was associated with a significantly lower risk of T2DM incidence [268]. Regarding its body weight regulation activity, Kim et al. carried out a meta-analysis of randomized control trials (21 trials, involving 940 participants). Results evidenced a significant reduction of body weight in dietary treatments, which contained a median intake of 132g/d (median duration of 6 weeks) compared to diets without pulse intake [269]. Meanwhile, Bahrami et al. showed that greater intake of legumes (OR: 0.73; 95% CI: 0.64-0.84), lentils (OR: 0.61; 95% CI: 0.46-0.78), and beans (OR: 0.35; 95% CI: 0.17-0.74) were associated with lower risk of NAFLD [264].

Several authors point out that the positive effect of legumes consumption on health may be attributed to the stating properties, fibre, vitamins, minerals and phytochemicals content [263] [270]. Starch is the main carbohydrate component in legumes and contains two types of polysaccharides, amylopectin and amylose (30-40% starch resistant), making it more resistant to gastrointestinal digestion [263]. The consumption of resistant starch has shown significant improvements in glycemic control and satiety, which could be used as potential weight-reducing agent [271]. Furthermore, legumes contain elevated concentrations of phenolic compounds that confer lipase, α-amylase and α-glucosidase inhibitory effects [270].

Dietary fibre seems to have a positive impact on the formation of short-chain fatty acids, contributing to the invasion intestinal microbiome balance [263]. Legumes also have soluble dietary fibre, including pectins, gums, inulin-type fructans and some kinds of hemicelluloses [263]. It has hypothesized that viscous soluble fibre could inhibit the cholesterol absorptive intestinal process [267,272]. Likewise, soluble fibre might reduce the secretion of

hepatic apolipoprotein B and consequently, play an essential role in the reduction of hepatic free cholesterol [272]. The up-regulation of genes related to β-oxidation and acetyl-CoA degradation and the down-regulation of glycolytic and lipogenesis genes could represent another mechanism that could explain the effects on metabolic profile [273]. Hence, higher legume consumption could be a functional food, and therefore it should be highlighted the possible benefits on metabolic disturbances related to NAFLD.

Based in our results, legume intake might hold benefits on the liver and cardiometabolic outcomes linked to NAFLD. However, further studies are needed to understand better the underlying biological mechanisms of these associations on NAFLD onset in individuals diagnosed with MetS.

Physical activity not only promotes weight reductions, but also cardiovascular and bone health, reduce muscle loss, preserve and strengthen muscle mass [181]. In ageing, sarcopenia is characterized by the reductions in skeletal muscle mass and strength, which are linked to chronic illness, insulin resistance, falls, and functional decline [274] and NAFLD [275]. In this sense, it is vital to prevent and reverse sarcopenia in the older population with a higher risk of NAFLD [274]. Nevertheless, despite the benefits of physical activity in health outcomes, there is not a consensus for NAFLD management. To improve our understanding of this issue, the association between physical activity and risk to develop NAFLD in older subjects diagnosed with MetS was examined. A highest tertile of physical activity (>61.4 to 321.7 MET-h/week) was associated with reduced HSI values compared to participants from the lowest tertile (<22.5 MET-h/week) of physical activity. Similar results have been reported by Kwak et al. where total physical activity was inversely associated with NAFLD independently of visceral obesity and insulin resistance [184]. Individuals who did ≥500 MET-min/week presented a 34% decreased risk of NAFLD compared with sedentary subjects [184]. On the one hand, a study revealed that individuals with vigorous physical activity had decreased odds of having NASH (OR: 0.65; IC: 0.43-0.98) [276]. On the other hand, doubling the

vigorous-intensity physical activity (75 minutes per week) was associated with decreased odds of fibrosis (OR: 0.53; IC: 0,29-0.97) [276]. Also, Kim et al. showed that moderate to vigorous physical and longer physical activity were linked to lower all-cause and CVD mortality in subjects with NAFLD [277].Our study also evidenced that physical activity is inversely correlated with the HSI. Accordingly, some studies have looked into the relationship between hepatic steatosis and exercise, reporting its improvements on the intrahepatic triglyceride content [190,278]. For instance, Sullivan et al. evaluated the effect of moderate-intensive exercise program recommended by the Department of Health and Human Services, the American College of Sports Medicine, and the American Heart Association on individuals with NAFLD [190]. Results demonstrated that participants who followed this exercise program presented a small decrease in the intrahepatic lipid content even when total body fat and body weight are maintained [190]. It has also been reported that vigorous exercise induced a significant reduction of 5% and moderate exercise a 4.2% of intrahepatic lipid content among individuals with NAFLD [278]. Furthermore, in a recent meta-analysis, authors showed that physical activity (aerobic or resistance exercise) had a lightly improvement on hepatic enzyme levels, serum lipid levels and intrahepatic lipid content in non-diabetic individuals diagnosed with NAFLD [279]. Our data corroborate the results from several studies performed in the PREDIMED-Plus trial [198,205,280,281]. In this context, sedentary behaviours such as greater time viewing TV, it was associated with a higher prevalence of obesity, abdominal obesity and T2DM [205]. In contrast, greater time spending in moderate-vigorous physical activity was associated with a lower incidence of obesity, central obesity, T2DM and lower levels of HDL-c [205]. Likewise, investigators reported that moderate-higher physical activity could have protective effects on sarcopenia, body fat composition, muscle strength [198], and decreased levels of proinflammatory markers [280]. Also, greater physical activity was positive associated with lower systolic blood pressure and reduced amounts of VAT [281]. These previous underlines demonstrate just the importance of physical activity, considering that our population our elderly population that present MetS and obesity, entities closely related to NAFLD risk.

The beneficial effects of regular physical activity on NAFLD may have been due to the increased glucose uptake from the circulation into the muscles and FFA uptake, the modulation of the β-oxidation [282], and the reduction of VAT [278], which is connected to increased flux of FFA into the liver trigger to hepatic steatosis [58]. In fact, it has been postulated that physical activity might revert some components associated with NASH (inflammation and histological features), and also mitochondrial functions [282].

Physical activity could stimulate adaptations in the electron transport chain and tricarboxylic acid cycle [282] [113]. Nonetheless, specific pathways are not widely understood [282]. It is fundamental to note that physical activity has a positive impact on metabolic profile, body composition, sarcopenia, and some pathophysiological features related to NAFLD in older subjects. Taking together, our data provide additional support for considerable insight into the importance of physical activity in risk factors associated with NAFLD.

Finally, our results are also supported by Salas-Salvadó et al. in the PREDIMED-Plus study. Results showed that intensive lifestyle intervention for 12 months was effective in reducing body fat composition, inflammatory markers, and promoted greater improvements on CVD risk factors [283]. With this in mind, our study provides additional further evidence to emphasize lifestyle behaviours are important modifiable risk factors for NAFLD in older individuals with MetS.

4. **Urine resveratrol profiling in cardiometabolic and liver outcomes**

An increased number of studies have found that resveratrol might have beneficial effects, modulating metabolic profile, inflammatory and oxidative pathways [113,156,164,165]. Nevertheless, in NAFLD, strong evidence from human clinical trials are still lacking, due to shortfall of well design clinical trials, statistical power and heterogeneity of the studies [143,284]. For this study, we used UPLC-Q-q-Q MS targeted metabolomics method, using authentic glucuronide and sulfated standards to quantify each resveratrol phase II metabolites (trans-

resveratrol-3-O-glucuronide, trans-resveratrol-4'-O-glucuronide, cis-resveratrol-3-O-glucuronide, cis-resveratrol-4'-O-glucuronide, and trans-resveratrol-3-O-sulfate). Remarkably, we found that participants with higher total urine resveratrol levels presented better lipid profile (triglycerides, VLDL-c and total cholesterol/HDL ratio) and liver enzymes compared (AST and GGT levels) to the lowest tertile.

Little is known about the association between resveratrol and NAFLD in individuals with MetS [138,142]. However, some clinical studies have reported improvements on glucose-insulin metabolism, lipid profile, and liver outcomes in individuals at higher risk of CVD and associated comorbidities [285–288]. A meta-analysis evaluated the effectiveness of resveratrol on cardiovascular risk factors in subjects with overweight or obesity [285].Results indicate that individuals with an intake of ≥300 mg/d of resveratrol exhibited significant reductions of total cholesterol, glucose, insulin and blood pressure [285]. Moreover, a meta-analysis showed that resveratrol supplementation (≥6 months with doses ranged from 8.1 to 3000 mg/d) could improve triglyceride concentrations in subjects with T2DM [288]. Similarly, in the PREDIMED study observed that increased urinary resveratrol metabolites were associated with lower triglycerides and higher HDL-c levels [287]. Nevertheless, our study did not found differences in the HDL-c levels. Possible explanations for this disagreement could rely on the effects of the synergetic effects of the anti-diabetic drug, lipid-lowering medication and resveratrol in T2DM condition, that could exert a synergic effect [288]. Also, the authors used resveratrol aglycone to quantify all glucuronide and sulfated metabolites, which are not well suitable for the quantification of resveratrol metabolites [289]. On the other hand, we did not find a significant association between total urine resveratrol metabolites and glucose parameters, but generally, the evidence of resveratrol effectiveness on glucose metabolism are ambiguous [290]. For instance, a recent meta-analysis revealed that resveratrol had positive effects on fasting plasma glucose and HOMA [291]. In contrast, a crossover randomized

controlled trial with 4 weeks of intervention (150 mg/day) did not show improvements in HOMA, glucose and insulin levels [292]. Another study no significant changes were observed in insulin resistance markers, liver enzymes, hepatic steatosis, and serum lipids in obese individuals with NAFLD, which received 3000 mg/d of resveratrol for 8 weeks [174]. Likewise, Akbari et al. suggested that resveratrol supplementation might significantly reduce total cholesterol, but did not changes on other lipid markers and liver enzymes in participants with MetS [293]. However, the conclusion of these studies should be interpreted carefully, contemplating that Cachay and investigators only included men in their study [174]. This point is important considering that women could be a higher activity of the UGT, which plays a crucial role in the metabolism of the resveratrol [294]

In the current analyses, flexible regression models were applied to assess the relationships between total urine resveratrol metabolites and risk of abnormal liver enzymes. This type of analysis is considered as a practical graphic tool, and also it could estimate the effect of a covariate, which has known large sample statistical properties [295]. Noteworthy, total urine resveratrol metabolites were associated with a lower risk of higher ALT, AST and GGT levels. In the same line, a study reported that NAFLD participants supplemented with resveratrol (500 mg/day of resveratrol for 12 weeks), exhibited reduced levels of ALT and hepatic steatosis. However, no significant changes in anthropometric, insulin resistance markers and lipid profile were found [173]. Besides, Chen et al. showed that resveratrol supplementation (600 mg/day for 3 months) could decrease levels of liver transaminases, LDL-c, total cholesterol and HOMA-IR in, but did not found a significant reduction in liver steatosis [175]. The reason for contradictory results on glucose, lipid and liver outcomes is still not completely clear, but the foremost cause of the discrepancy is due to lower doses of resveratrol less could not have a positive effect on glucose levels [172]. Also, chronic higher doses of resveratrol could stimulate the saturation in absorption sites [152,174]. Another plausible explanation is that inter-individual variations as a result of sex, age, microbiota, lifestyle, genetics, and other factors could be playing a crucial role in the effectiveness of resveratrol on health outcomes [296,297]. It is thus also necessary to take into

account that the resveratrol content varies in the same type of fruit, grape, and wine leading to the low bioavailability [298,299].

Many mechanisms implicated in the effectiveness of resveratrol on health have postulated [162,165,300,301]. It was pointed out that resveratrol metabolites could be incorporated into LDL after intake of moderate red wine [162]. This is important to highlight that resveratrol could have positive effects on lipid metabolism. About liver outcomes, resveratrol might be involved in the activation of AMPK leading to the inhibition of the SREBP-1 activity [300], the regulation of hepatic enzyme 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) related to cholesterol synthesis [165], and the overexpression of the paraoxonase 1 (PON 1) [301].

Altogether, our results further our knowledge of the relationship between resveratrol and NAFLD risk factors in individuals diagnosed with MetS. However, there is a critical need for extensive clinical large studies investigating the mechanism underlined on the effects of resveratrol in this population.

5. Strengths and limitations

The main strengths of this study are a robust large sample size within the PREDIMED Plus trial with detailed information related to dietary intake and patterns, sociodemographic, non-invasive liver markers, clinical and biochemical data. Moreover, the study was carried out in older patients diagnosed with MetS considering that this clinical condition is strongly related with increased risk to develop non-communicable diseases such as CVD and NAFLD, which represent the leading cause of mortality and disability worldwide.

It should be emphasized the use of valuable approaches such as the DXA for body composition measurements, and metabolomics using an adequate analytical method and authentic standards for identifying and quantifying resveratrol metabolites. Finally, we applied multiple models adjusted by potential confounders, which minimize the effects of these on the principal outcome. However, this research has some limitations that should be declared:

First, the cross-sectional and non-prospective design, which a causal relationship cannot be inferred causality, but this type of study design provides preliminary data about possible relationships to support further research.

Second, liver biopsy was not performed for diagnosing NAFLD. Participants were assessed using several validated non-invasive liver markers, which have been extendedly performed in large-epidemiological studies [67,68,92,94,219].

Third, in the analysis of urine resveratrol metabolites, we did not use all phase II metabolites, glucosides and gut microbial metabolites. Thus, our results could be influenced. However, we included resveratrol glucuronide and sulfate conjugates that the majority of human studies have been reported in plasma and urine samples [298 302]. The detection of other metabolites remain complex leading to heterogeneous conditions, doses and the use of different analytical methods in human trials. Also, it should be considering the limited availability of authentic standards challenging the quantification of resveratrol metabolites.

Fourth, the use of the FFQ for the dietary assessment tend to be usually under-reported, but the current FFQ were previously validated in Spanish population [206] and collected by trained registered dietitians during face-to-face visit [195 196].

Fifth, our study sample focused on Caucasian older individuals that limit the extrapolation of our results to other populations. Nevertheless, this research includes individuals with increased metabolic risk factors, considering that these conditions are prevalent in Western countries.

6. General overview

This thesis evidences that biological sex-related differences could be involved in the predisposition to NAFLD. Moreover, individuals with insulin resistance measured by the TyG index and expanded VAT presented atherogenic risk profile and adverse liver outcomes closely related to NAFLD development. Interestingly, the TyG index, recognized as a novel indicator of insulin resistance, might be proposed as a proxy marker of dysfunctional VAT phenotype in women diagnosed with MetS.

Our research also widens our knowledge of lifestyle behaviours on NAFLD risk factors. Noteworthy, individuals with greater adherence to the MedDiet and highest physical activity showed lower levels of HSI. In this context, dietary modifications and physical activity could be ideal for the management of NAFLD.

The beneficial effects of the MedDiet on metabolic disorders are mainly recognized but not only for the higher intake of healthy dietary components but also for the consumption of polyphenols rich-foods. In this research, it was found a significant inverse association between higher intake of legumes and increased HSI values. Moreover, subjects with higher total urine resveratrol metabolites exhibited a better lipid profile and liver enzymes. Likewise, urine resveratrol concentrations were associated with a reduced odds ratio for abnormal levels of liver enzymes (above the upper limit of normal).

VI.   CONCLUSIONS

CONCLUSIONS

1. Women had increased risk to develop NAFLD measured by the hepatic steatosis index (HSI) compared to men, which suggest the potential role of sex differences on risk factors related to NAFLD in elderly individuals.

2. Individuals who presented higher VAT deposition exhibited a significant association with atherogenic dyslipidaemia, abnormal glucose metabolism, and liver impairments characterized by increased FLI, HSI, FGF-21 and ALT levels in subjects diagnosed with MetS. These results offer compelling evidence that differences in the distribution of VAT might be a strong contributor to adverse cardiometabolic and liver parameters linked to NAFLD in subjects with MetS features.

3. A positive association between higher TyG and non-invasive liver markers such as ALT, GGT, FGF-21, FLI, and HSI suggest that insulin resistance contributes to the development of NAFLD in subjects who were overweight or obese at high CVD risk. In addition of being an insulin resistance marker, the TyG index could be incorporated as a reliable and simple indicator of an enlarged VAT deposition in women, favouring healthcare professionals in the screening of subjects with MetS manifestations.

4. A greater adherence to the MedDiet and higher physical activity were inversely associated with HSI values as related to liver status, supporting that lifestyle modification could be a primary therapy in the prevention and management of risk factors linked to NAFLD.

5. A higher intake of legumes was associated with a lower risk for showing elevated HSI values, suggesting that legume consumption may be an essential aspect of the Mediterranean dietary pattern that could provide benefits on liver health outcomes.

6. Participants with higher urinary resveratrol metabolites exhibited better lipid profile (triglycerides, VLDL-c, and total cholesterol/HDL ratio) and lower levels of serum enzymes (AST and GGT levels). Furthermore, total urine resveratrol excretion was linked to a lower risk of having liver enzymes above the reference normal limit, indicating that some resveratrol metabolites might be involved in the modulation of the risk factors associated to NAFLD.

General conclusion

The implications of sex-specific differences and individual phenotype are relevant factors on the risk to develop NAFLD. This relationship was even more marked in individuals with an adiposopathy VAT and insulin resistance features. Also, greater adherence to the MedDiet with some dietary components such as legumes, specific bioactive compounds (resveratrol), and physical activity showed beneficial effects on liver outcomes, which could be considered as part of a liver-healthy lifestyle in older individuals with a higher risk of NAFLD and MetS manifestations.

VII.   REFERENCES

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VIII. ANNEXES

Bullón-Vela M, Abete I, Martinez JA, Zulet A (2019). Obesity and Nonalcoholic Fatty Liver Disease: Role of Oxidative Stress. En: Marti del Moral, Amelia; Aguilera, Concepción M. (ed.). Obesity: Oxidative Stress and Dietary Antioxidants, Academic Press, pp. 111-133. ISBN: 9780128125045. https://doi.org/10.1016/B978-0-12-812504-5.00006-4

Fibroblast growth factor 21 levels and liver inflammatory biomarkers in obese subjects after weight loss

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Abstract

Introduction: Previous studies have hypothesized fibroblast growth factor 21 (FGF-21) as a potential biomarker of the inflammation associated with liver diseases, which is also receiving considerable attention for its potential application concerning the management of obesity and co-morbidities. This study aimed to analyze the response of FGF-21 after a weight loss intervention and the relationships with other putative inflammatory liver biomarkers.

Material and methods: Sixty-six obese participants from the RESMENA study were evaluated at baseline and following a 6-month energy restriction treatment. Anthropometric, body composition by DXA, routine laboratory measurements, which included transaminases and gamma-glutamyl transferase (GGT) were analyzed by standardized methods. Moreover, FGF-21, M30 fragment (M30) and plasminogen activator inhibitor-1 (PAI-I) were analyzed as recognized liver inflammatory related biomarkers with specific ELISA kits.

Results: Most measurements related to hepatic damage, inflammation and adiposity status improved at the end of the 6-month nutritional intervention. In addition, ΔFGF-21 shifts showed statistical relationships with changes in ΔM30, ΔGGT and ΔPAI. The reduction of M30 showed significant associations with changes in transaminases. Furthermore, PAI-I changes were associated with ΔM30 and ΔGGT regardless of weight loss. A linear regression model was set up to assess the influence of ΔPAI-I and ΔM30 on the variability of ΔFGF-21 (23.8%) adjusted by weight loss.

Conclusions: These results demonstrated interactions of some liver inflammatory mediators, specifically M30 and PAI-I with FGF-21. Thus, more investigation about FGF-21 is required given that this protein could be a biomarker of the obesity-inflammation-liver process.

Key words: CK-18, NAFLD, M30, obesity, metabolic syndrome, inflammation.

Introduction

Obesity rates are increasing in developed and transition countries, becoming a worldwide epidemic [1]. In 2025, if the prevalence growth con-

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tinues with this trend, the global figures of obesity will reach 18% in men and 21% in women [2]. In addition, it is well known that obesity is commonly accompanied by different comorbidities such as type 2 diabetes, hypertension, dyslipidemia, cardiovascular disease, and non-alcoholic fatty liver disease (NAFLD), among others [3]. The prevalence of NAFLD is rising in parallel with the obesity epidemic, being worldwide one of the most important chronic liver diseases [4]. Currently, NAFLD is considered as a frequent component of the metabolic syndrome, which is closely related to inflammatory processes [5, 6]. The mechanisms underlying the progression of this disease are still largely unknown [7]. In any case, a number of studies have identified diverse NAFLD multifactorial determinants and causal interactions with genetic factors, oxidative stress, hormones secreted from the adipose tissue and systemic inflammation as well as unbalanced nutrition and unhealthy lifestyles [8]. Nowadays, the investigations are focusing on a novel liver biomarker, which has led to an important evolution over the past 8–10 years regarding suitable non-invasive diagnostic tools for fatty liver. However, most scores and indexes are not still fully valid in clinical practice [9]. Some studies have suggested M30 fragment (CK-18) and FGF-21 as potential biomarkers for NAFLD [10]. M30 fragment estimates hepatocyte apoptosis in NAFLD or non-alcoholic steatohepatitis (NASH), which is related to inflammation mechanisms and fibrosis stages [11, 12]. Another study [13] even suggested that CK-18 fragments (formed by M30 and M65 fragments) might be a useful biomarker to discriminate between NAFLD and NASH conditions. For instance, a recent review highlighted CK-18 as one of the more promising biomarkers to diagnosis NAFLD [14]. On the other hand, fibroblast growth factor 21 (FGF-21) is released from liver cells, from adipocytes and in other tissues [15]. A functional role for FGF-21 is to maintain the glucose and lipid homeostasis [16]. Previous studies have reported that in over-nutrition as well as in inflammatory processes, FGF-21 levels are increased [17]. Anti-inflammatory effects have been demonstrated to have beneficial results in obesity and comorbidities [18, 19]. In addition, levels of FGF-21 are elevated in NAFLD subjects and some investigators have found that FGF-21 is an independent biomarker to predict NAFLD [20]. Nowadays, the "gold standard" test to diagnosis NAFLD is the liver biopsy, for steatosis, fibrosis or cirrhosis [21]. Nevertheless, it is an invasive and unreliable procedure for many patients and therefore it is not frequently performed [22]. In this context, the treatment of NAFLD is based on general dietary weight loss strategies, with a lack of information about specific dietary approaches through

well-designed randomized trials [23]. In this context, it is necessary to find suitable non-invasive biomarkers which, alone or in combination with other scores, are able to provide information to monitor metabolic disease such as NAFLD management in a personalized way [24, 25]. The present study aimed to evaluate the response of FGF-21 circulating levels to a weight loss intervention and their relationships with metabolic and inflammatory liver biomarkers such as the recently proposed liver disease biomarkers M30 fragment (CK-18 fragments) and PAI-1, in obese patients with metabolic syndrome.

Material and methods

Study design

The current study enrolled 66 participants of the RESMENA-S study, which was designed as a randomized, controlled intervention trial to compare the effects of two hypocaloric dietary strategies (American Heart Association and RESMENA) on metabolic syndrome features after a six-month follow-up. Subjects were assigned (using the "random between 1 and 2" function in the Microsoft Office Excel 2003 software (Microsoft Iberica, Spain) to follow one of the two energy-restricted diets described elsewhere [26]. Both diets have been found to produce similar weight loss and metabolic outcomes, so both dietary groups were merged for the current analyses as statistically designed in other investigations of the RESMENA study [27]. Physical activity was evaluated by a 24 h questionnaire at the beginning and at the end of the study [26].

The RESMENA study followed the CONSORT 2010 guidelines, except for blinding. This research was performed according to the ethical guidelines of the Declaration of Helsinki, and it was registered. The trial was approved by the Research Ethics Committee of the University of Navarra (ref. 065/2009). Additional aspects of this intervention trial have been detailed elsewhere [28].

Nutritional intervention

Two hypocaloric dietary strategies, both with the same energy restriction (-30% of the individual's requirements), were prescribed and evaluated. The reference diet was based on American Heart Association (AHA) guidelines, including 3–5 meals/day, a macronutrient distribution of 55% of total caloric value (TCV) from carbohydrates, 15% from proteins and 30% from lipids, a healthy fatty acid (FA) profile, a cholesterol consumption lower than 300 mg/day and a fiber intake of 20–25 g/day. Otherwise, the RESMENA diet was designed with a higher meal frequency, consisting of 7 meals/day, and a macronutrient distribution of 40% TCV from carbohydrates, 30% from

proteins and 30% from lipids. The RESMENA diet also maintained a healthy fatty acid profile rich in omega-3, extra virgin olive oil intake, a cholesterol content of less than 300 mg/day and a fiber intake of 20–25 g/day. A 48-h weighed food record was completed at the beginning and at the end of the study, which was used to evaluate the volunteer's adherence to the prescribed diet. The energy and nutrient content of these questionnaires were determined with the DIAL software (Alce Ingenieria, Madrid, Spain), as described elsewhere [26]. Anthropometric determinations were measured in fasting conditions and following standardized procedures previously reported [28]. Body composition was assessed by dual-energy x-ray absorptiometry (Lunar Prodigy, software version 6.0, Madison, WI), at baseline and at the end of the trial, according to validated protocols [26]. Body mass index (BMI) was calculated as the body weight divided by the squared height (kg/m^2). Glucose, total cholesterol (TC), triglycerides (TG), ALT, AST, and GGT were measured on an autoanalyzer (Pentra C-200; HORIBA ABX, Madrid, Spain) with specific kits from this company. Plasma concentrations of CK18 fragment (M30) levels were assessed by ELISA assay (PEVIVA, Nacka, Sweden) with an autoanalyzer system (Triturus, Grifols SA, Barcelona, Spain) following the manufacturer's instructions. FGF-21 concentrations were analyzed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biovendor, Brno, Czech Republic) according to the supplier guidelines [29]. Fasting plasma levels of leptin and adiponectin were measured by ELISA using commercially available kits (Millipore, MA, USA). Plasma concentrations of high-sensitivity CRP (hsCRP) (Demeditec) were measured, and IL-6 (R&D Systems), TNF-α (R&D Systems), and PAI-I (BioVendor) were measured with specific enzyme-linked immunosorbent assay kits from the specified suppliers using an autoanalyzer system (Triturus, Grifols SA, Barcelona, Spain) in accordance with the manufacturer's instructions. Homeostatic model assessment of insulin resistance (HOMA-IR) and homeostatic model assessment of β cell function (HOMA-β) were estimated using the equation of Matthews *et al.* [30]. The fatty liver index (FLI) [31] is an algorithm derived from serum TG, BMI, WC and GGT levels [32–35], which was validated in a large group of subjects with or without suspected liver disease with an accuracy of 0.84 (95% CI) in detecting a fatty liver condition. FLI varies between 0 and 100, the presence of steatosis being estimated when FLI score ≥ 60.

Statistical analyses

Analyses were performed using STATA version 12.0 (Stata Corp). According to previous studies and in the current investigations both diets (AHA and RESMENA) were equally beneficial and effective concerning weight loss and metabolic outcomes. For these the data were analyzed as one group. Changes in given variables were calculated as values of the end of the intervention less values at baseline. To analyze the changes of inflammation biomarkers during the intervention, the median value of the changes was used as a cut-off (above and below the 50th percentile) as previously applied [36]. Normality distributions of the evaluated variables were determined by means of the Shapiro-Wilk test. Continuous variables were compared between groups by Student's *t*-test or the Mann-Whitney *U* test for parametric or non-parametric variables, respectively. The relationships among variables were assessed by Pearson's correlation coefficient or Spearman's rho (*p*), as appropriate depending on normality distributions. Multivariate linear regression models were fitted to assess the potential influence of liver-inflammation factors on FGF-21 levels after treatment and adjusted for age and gender. Two regression models were performed. Model 1 included changes after treatment in M30 and PAI-I. Model 2 also included changes in body weight. All *p* values presented are two-tailed, and differences were considered statistically significant at *p* < 0.05.

Results

A total of 109 Caucasian adults were enrolled to participate in this study. Then, 93 participants started the dietary interventions after inclusion screening. They showed no statistically significant differences concerning anthropometric and biochemical measurements at the end of the 6 months. During follow-up there were patients who withdrew due to lack of adherence of the informed consent. The present study evaluated a subsample of 66 subjects (50.8 ±8.6; 36 M/30 F) who completed all the required data. Changes in anthropometric parameters and body composition as well as lipid and glucose metabolism values were assessed by standard procedures at baseline and after weight loss (Table I) in order to screen the general outcomes of the intervention. All measurements significantly improved at the end of the intervention. In addition, in order to characterize the evolution of hepatic markers and related liver inflammatory biomarkers paired *t*-tests were performed at baseline and after the nutritional follow-up. All hepatic and inflammatory markers also showed a significant clinical amelioration (Table II). In that sense, several linear regression analyses of data changes in FGF-21 and other inflammatory liver markers after the intervention period were fitted with the purpose of discriminating the relation with M30 (cell apoptosis marker) and PAI-I

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Table I. Anthropometric and body composition, lipid and glucose metabolism of the participants after 6 months of dietary intervention to lose weight

Parameter	Baseline	6 months	*P*-value
Anthropometric and body composition			
Weight [kg]	99.4 (15.8)	90.4 (15.5)	< 0.001
BMI [kg/m²]	35.7 (43.0)	32.6 (4.5)	< 0.001
Android fat mass [kg]	4.6 (1.2)	3.8 (1.5)	< 0.001
Total fat mass [kg]	41.5 (9.2)	34.5 (9.1)	< 0.001
Lipid and glucose metabolism			
Glucose [mg/dl]	124.7 (38.1)	113.4 (28.7)	0.006
Total cholesterol [mg/dl]	388.7 (153.0)	278.3 (120.0)	< 0.001
HDL cholesterol [mg/dl]	45.4 (90.0)	48.8 (10.1)	0.001
LDL cholesterol [mg/dl]	142.6 (39.2)	172.5 (38.4)	< 0.001
TG [mg/dl]	177.8 (92.0)	136.4 (77.0)	< 0.001
Insulin [U/l]	13.2 (7.1)	7.9 (5.8)	< 0.001
HOMA-IR	4.1 (2.5)	2.3 (2.0)	< 0.001

Mean ± SD. Paired t-test was carried out. P < 0.05 was considered statistically significant. BMI – body mass index, VAT – visceral adipose tissue, HDL – high-density lipoprotein, LDL – low-density lipoprotein, TG – triglycerides, HOMA-IR – homeostatic model assessment insulin resistance.

Table II. Hepatic and inflammatory markers of the participants after 6 months of dietary intervention to lose weight

Parameter	Baseline	6 months	*P*-value
Hepatic markers			
ALT [U/l]	33.4 (17.8)	24.5 (8.8)	< 0.001
AST [U/l]	24.2 (9.4)	21.5 (5.2)	0.029
GGT [U/l]	42.1 (26.0)	24.2 (16.5)	< 0.001
FLI	86.99 (14.1)	68.1 (3.2)	< 0.001
M30 [U/l]	183.7 (129.5)	118.3 (49.4)	< 0.001
Inflammatory markers			
Leptin [ng/ml]	20.8 (15.3)	15.5 (13.8)	0.001
Adiponectin [ng/ml]	13.1 (10.0)	16.2 (12.4)	0.002
hs-CRP [mg/dl]	3.3 (3.2)	2.1 (2.4)	0.001
IL-6 [pg/ml]	2.4 (1.5)	1.8 (1.2)	< 0.001
PAI-I [pg/ml]	173.4 (111.3)	108.9 (64.1)	< 0.001
FGF-21 [pg/ml]	184.9 (165.3)	91.9 (93.7)	< 0.001

Mean ± SD. Paired t-test was carried out. P < 0.05 was considered statistically significant. ALT – alanine transaminase, AST – aspartate transaminase, GGT – gamma-glutamyl transferase, FLI – fatty liver index, M30 – M30 fragment of CK18, hs-CRP – high-sensitivity C-reactive protein, IL-6 – interleukin 6, PAI-1 – plasminogen activator inhibitor-1, FGF-21 – fibroblast growth factor 21.

(plasminogen activator inhibitor). Some variables were independently studied by univariable linear regression. Therefore, variables potentially associated with FGF-21 were: Δ M30 (β = 0.26, *R* = 0.048, *p* = 0.066), Δ PAI-I (β = 0.665, *R* = 0.239, *p* < 0.001), Δ Age (β = –25.2, *R* = –0.008, *p* = 0.458). When these variables were jointly considered, the pre-

dictors of the model explained up to 24.1% (Table III) of the variation of the Δ FGF-21 in model 1 (adjusted R^2 = 0.241, P_{model} = 0.008). Likewise, the energy restricted nutritional intervention and subsequent weight lowering could affect this biomarker. Thus, in model 2 (Table III) the linear regression with the same outcomes was adjust-

Table III. Linear regression analyses according to changes in fibroblast growth factor 21 (FGF-21) involving different inflammatory liver markers after the 6-month intervention

Model 1	β-coefficient	IC	*P*-value
Δ M30	0.72	0.18 to 1.25	0.039
Δ PAI-I	0.66	0.31 to 1.00	< 0.001
P-model (*R*²-adjusted)			0.008 (0.241)
Model 2	**β-coefficient**	**IC**	***P*-value**
Δ M30	0.66	0.11 to 1.22	0.041
Δ PAI-I	0.63	0.27 to 0.98	0.001
Δ Weight loss	−3.13	−10.54 to 3.27	0.293
P-model (*R*²-adjusted)			0.008 (0.238)

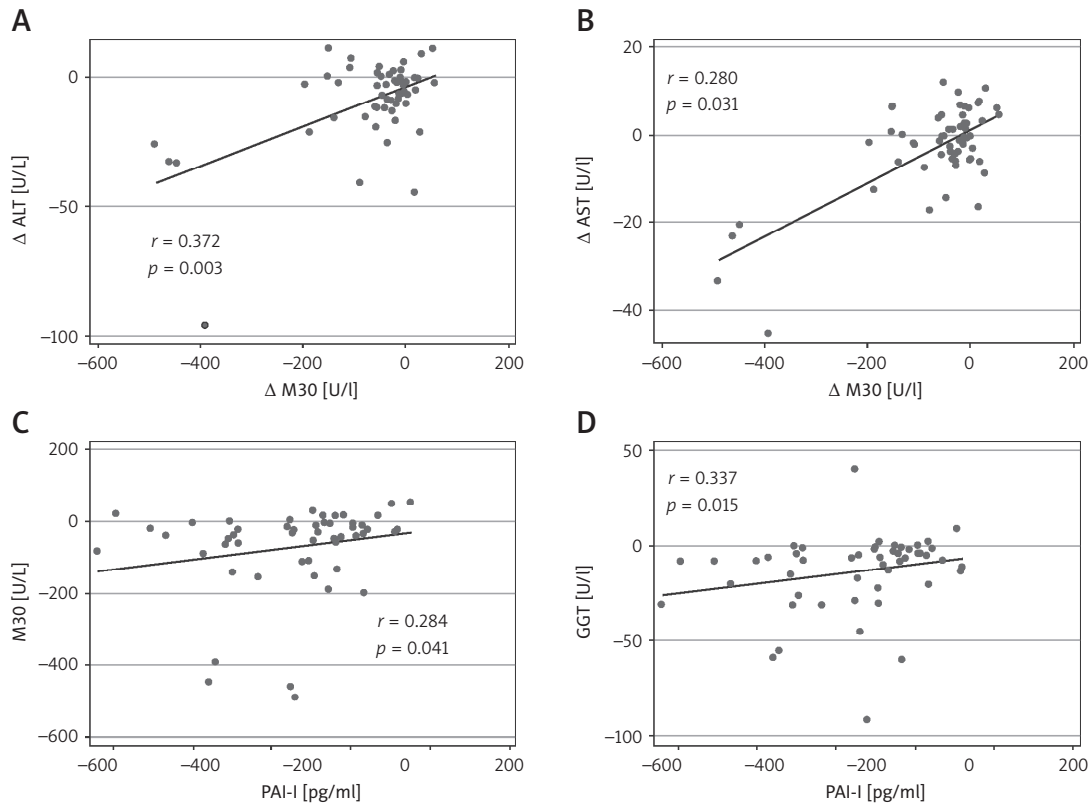*Models 1 and 2 were adjusted by age and gender. M30 – M30 fragment of CK18, PAI-I-I – plasminogen activator inhibitor-1.*

ed by Δ of weight loss, evidencing that the effect of variations of the inflammatory markers on FGF-21 is independent of the weight loss processes. In this context, the relationships between hepatic markers and inflammatory parameters were studied with the objective of seeking relevant clinical information. Thus, changes in FGF-21, M30 fragment and PAI-I as non-invasive liver markers with inflammatory biomarkers and metabolic status are also reported (Figures 1 and 2). Positive and significant associations were found between FGF-21 and M30 ($r = 0.346$; $p = 0.012$), GGT ($r = 0.409$; $p = 0.003$) and PAI ($r = 0.521$; $p < 0.001$). In addition, M30 showed significant relations with ALT ($r = 0.372$; $p = 0.003$) and AST ($r = 0.280$; $p = 0.031$). On the other hand, PAI-I showed significant associations with GGT ($r = 0.337$; $p = 0.015$) and M30 ($r = 0.284$; $p = 0.041$). Finally, in order to reinforce these results, Figure 3 illustrates that in accordance with P_{50} of Δ M30 and Δ PAI-I, the largest reductions in both inflammatory markers were linked with the largest decreases in Δ FGF-21.

Discussion

NAFLD is becoming one of the most common liver diseases worldwide. Its complex pathogenesis is closely associated with metabolic disorders such as obesity [3, 37, 38]. To understand the specific mechanisms involved in this process is an urgent necessity [39]. Nowadays, molecules such as FGF-21 have emerged as potential players or biomarkers of the NAFLD pathogenesis in a framework of inflammation processes [40]. In this context, this study showed that the improvement in hepatic-inflammatory biomarkers induced by an energy restriction treatment prescribed to lose weight is associated with a concomitant reduction in FGF-21 circulating levels. Relevantly, this association was not directly related to weight loss, suggesting that the improvement in the inflamma-
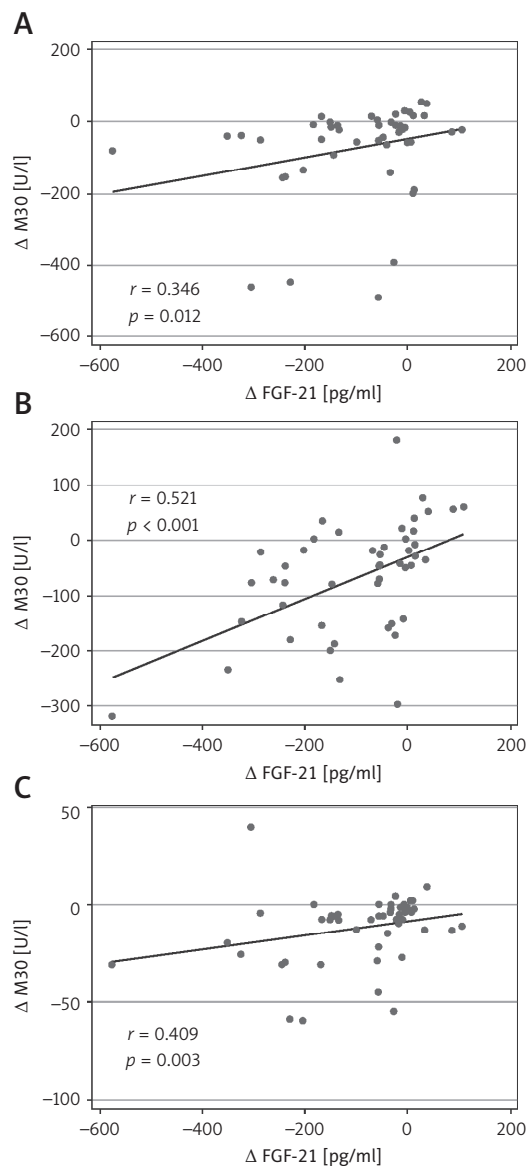
Figure 1. Correlations of changes of fibroblast growth factor 21 (FGF-21) with changes of M30 (CK-18 fragment), plasminogen activator inhibitor (PAI-I) and gamma-glutamyl transferase (GGT)

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A

B

C

D

Figure 2. Associations between changes of inflammatory related hepatic markers
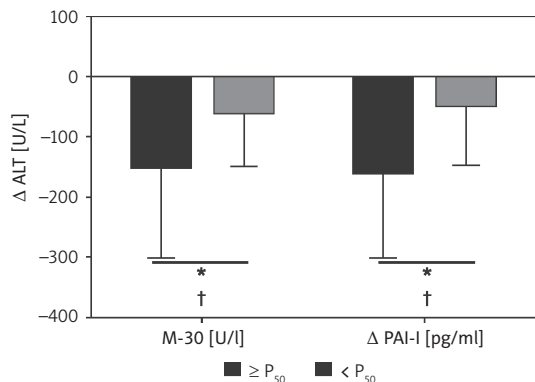
Figure 3. Changes in fibroblast growth factor 21 (FGF-21) according to the magnitude of changes in M30 and plasminogen activator inhibitor (PAI-I) categorized for median (≥ p50 or < p50). *P < 0.05 was considered statistically significant. +P < 0.05 was considered statistically significant after adjustment for changes in body weight

tion induced by the dietary treatment is the most likely mechanism involved in the variation of FGF-21. Therefore, the current results highlight the relevance of FGF-21 as a non-invasive marker for prediction and management of liver disease. Several lines of evidence suggest that increased stress oxidation changes in several molecular factors, including adipokines, chemokines, and pro- or anti-inflammatory cytokines, are mainly involved in the progression of NAFLD/NASH [41]. Indeed, inflammation appears as a common fea-

ture with different stages of diverse liver disease, where specific assessment may help to better understand the progression of liver disturbances with specific inflammatory biomarkers such as FGF-21 or M30-frgaments. Regarding NAFLD treatment, dietary and lifestyle modifications are considered the first-step therapy for patients with NAFLD, but there is no consensus for pharmacological treatment [42]. It is important to take into account that this pathology has different grades of severity, which makes it necessary to develop appropriate biomarkers for its different stages [43]. For these reasons, current research has focused on identifying biomarkers to predict NASH or NAFLD [14, 44]. In this context, Bedogni *et al.* designed a simple scoring system named FLI, which includes TG, GGT, BMI, and WC, and is easily calculated. FLI was developed for the prediction of fatty liver disease (AUC = 0.84) [32]. The accuracy of FLI in comparison with the ultrasonography method for detection and quantification of hepatic steatosis has been validated in several countries [32]. Furthermore, previous studies have demonstrated and validated other non-invasive markers of liver status that were used in this investigation (M30 fragments) [45]. In addition, changes in recognized biochemical parameters, such us transaminases levels, are the most common non-invasive methodology to assess fatty liver. However, in patients with NAFLD more than 70% have normal liver enzyme values [46].

Early diagnosis of NAFLD is very important, but there is no single biochemical marker for the confirmation of NAFLD [47]. In this study, we evaluated the relationships between FGF-21 and M30 fragment (two inflammatory related molecules) with anthropometric and metabolic status markers in overweight/obesity subjects under energy restriction, using measurements at baseline and at the end of the intervention (6 months). As expected, the metabolic hepatic and inflammatory outcomes improved in most cases. Interestingly, inflammatory markers (FGF-21, M30 and PAI-I) indicated different clinical outcomes. First, FGF-21 was associated with markers of cell apoptosis (M30) and transaminases in our study. This marker has been shown as an independent predictor of NAFLD and inflammatory processes [48] in humans [20, 31, 49]. Other authors have suggested that FGF-21 can be used for early identification of hepatic steatosis [50]. Thus, circulating FGF-21 levels in humans have been found increased in pathologies such us obesity, metabolic syndrome insulin resistance and cardiovascular disease [51, 52]. Also, another study [53] demonstrated that FGF-21 was associated with hs-CRP, whose values were significantly elevated in patients with NAFLD and diabetes. On the other hand, several authors have demonstrated the relation of insulin resistance and diabetes with FGF-21, as occurred in our analysis [40]. In other words, FGF-21 has been related with processes of inflammation in NAFLD [54] and FGF-21 could have a compensatory effect to protect the body from adverse metabolic responses. The findings in human studies suggest that serum FGF-21 level has the potential to be an important biomarker for the early diagnosis of metabolic diseases or its complications [51]. Secondly, a study from Kawanaka et al. concluded that serum CK-18 (M30 fragment) levels can predict values of NASH in NAFLD patients [50]. Furthermore, it has been reported that CK18, including M30 and M65 fragments, is predictive of the prognosis of NAFLD [55]. However, another study suggests that plasma CK-18 has a high specificity for NAFLD and fibrosis, but its limited sensitivity makes it inadequate as a screening test for staging NASH [56]. Thus, it has been suggested that combined with other biomarkers such as FGF21, CK18 might be a complementary marker in non-invasive identification of NAFLD patients [50]. Third, PAI-I metabolism and functions are still scarce, although some studies have suggested that these cytokines could have a later effect in the obese state [57]. In a study from Thiruvagounder et al. increased levels of PAI-I showed significant associations with NAFLD and impaired fibrinolytic activity [58]. Contrarywise, in our study PAI-I showed a significant reduction at the end

of the intervention and PAI-I revealed significant positive associations with M30 fragment, GGT and FGF-21. Finally, according to the regression analysis in this study, it was found that when adjusted for changes in weight loss, the inflammatory markers are not affected. A limitation of this study is that NAFLD was evaluated using non-invasive markers instead of imaging techniques and/or liver biopsies. Also, the sample size of the current study is relatively small, and conclusions should not be extrapolated to the general population. Although type I error cannot be excluded, the obtained results reveal that the outcomes are concordant with the adopted hypothesis. Moreover, the design of the current trial is based on validated non-invasive and affordable markers, which makes them a suitable form of diagnosis in clinical practice. In addition, FLI was assessed in subjects at baseline and the whole sample presented moderate (FLI ≥ 30 and ≤ 60) and severe (FLI > 60) fatty liver. One of the main strengths of the present research is that it is a randomized controlled trial, considered the gold standard in the hierarchy of research designs for evaluating the efficacy and safety of a treatment intervention. Furthermore, the fact that every dietary pattern has been personally designed for each patient taking into account the sex, height, initial body weight and physical activity should also be highlighted. Finally, it is important to point out that a well-recognized healthy dietary pattern (AHA) was used as a comparative test, which shows that positive results obtained with the RESMENA diet should be considered of reasonable importance. As a corollary, the current research indicates that FGF-21 is sensitive to nutritional stress regardless of weight lowering, and that the mechanism of inflammation can be independent of weight loss.

In conclusion, the current study demonstrates that FGF-21 levels are modulated after an energy-restriction treatment in metabolic syndrome-obese patients concomitantly with an improvement in inflammation, hepatic damage and body composition. Importantly, the FGF-21 changes exhibited a strong association with M30 fragment and PAI-1, two non-invasive markers of liver inflammation. This association is independent of weight loss. Therefore, further investigation of FGF-21 is required since this molecule appears to be involved in the obesity-inflammation-liver process, and its plasma concentrations could add relevant information in the prediction and management of the liver pathogenesis related to obesity.

Conflict of interest

The authors declare no conflict of interest.

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