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SARCOPENIA, LIVER AND CARDIOVASCULAR ALTERATIONS IN A MULTICENTRIC COHORT OF PATIENTS WITH MASLD: EVALUATION OF CLINICAL PRESENTATION AND GENETIC PREDISPOSITION

TESI DI DOTTORATO DI ANNALISA CESPIATI

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ABSTRACT

Background and Aims: Sarcopenia, characterized by the progressive loss of skeletal muscle mass (SMM), strength, and function, is not solely an age-related condition but is also associated with several diseases, including chronic liver disease (CLD) and cardiovascular (CV) disease. Metabolic-dysfunction associated steatotic liver disease (MASLD) is defined by the presence of liver steatosis combined with, at least, one cardiometabolic risk factor, with liver fibrosis serving as a critical determinant of liver-related and CV-related events and mortality. Sarcopenia is highly prevalent in advanced MASLD, although recent studies have highlighted myosteatosis, the accumulation of fat within muscle, as a key driver of liver disease progression. Studies conducted mainly in Asia have reported a high prevalence of sarcopenia even in early MASLD, though it is not routinely assessed. Both sarcopenia and MASLD contribute to atherosclerotic CV damage, especially in the presence of advanced liver fibrosis, and share common pathogenetic mechanisms such as insulin-resistance (IR), low-grade chronic systemic inflammation, mitochondrial dysfunction, and oxidative stress. Additionally, genetic predisposition plays a role in MASLD, with specific polymorphisms in PNPLA3, TM6SF2, MBOAT7, and GCKR promoting severe liver disease, while the HSD17B13 variant appears protective against MASLD. Despite this background, most studies evaluating the relationship between sarcopenia, MASLD, and CV disease are cross-sectional, primarily involving Asian populations, limiting the generalizability of findings and the ability to determine whether the relationship is bidirectional. Furthermore, the absence of standardized criteria for diagnosing sarcopenia in MASLD complicates comparisons across studies, leading to conflicting results. To date, no studies have yet explored the role of genetic variants predisposing to MASLD in the risk of developing sarcopenia and CV damage. This project aims to address these gaps by 1) evaluating the impact of sarcopenia on liver and CV damage in non-cirrhotic MASLD patients, 2) investigating the influence of MASLD-related genetic variants on muscle mass and CV damage, and 3) assessing changes in muscle, liver, and CV parameters over a 5-year follow-up in a subset of patients.

Methods: 856 non-cirrhotic MASLD patients were recruited from liver outpatient clinics in Milan, Palermo, and London. At enrollment, anthropometric, demographic, and laboratory data were collected. Liver steatosis was assessed using abdominal ultrasound (US) and controlled attenuation parameter (CAP) measurements via Fibroscan, while liver fibrosis was evaluated using liver stiffness measurement (LSM) at Fibroscan. Severe steatosis was defined as CAP > 280 dB/m, and advanced fibrosis was identified as LSM ≥ 8 kPa. Liver biopsies were performed on 370 patients. Sarcopenia was assessed using bioelectrical impedance analysis (BIA), a non-invasive and radiation-free technique, with the skeletal muscle index (SMI) calculated as SMM/height^2. Patients were divided into gender-specific tertiles based on SMI (lowest, middle, and highest). CV damage was evaluated using carotid doppler US, with carotid intima-media thickness (cIMT) ≥ 0.9 mm or carotid plaques indicating CV damage. In 502 patients, epicardial fat thickness (EFT) was measured via echocardiography, and carotid-femoral pulse wave velocity (cfPWV) via tonometry. EFT ≥ 9.5/7.5 mm in men/women was considered a marker of visceral adiposity, while EFT ≥ 5.2 mm was associated with coronary artery disease. cfPWV > 10 m/sec was used as a marker of arterial stiffness. CV risk was calculated for all patients using SCORE2 and SCORE2-OP algorithms according to European Society of Cardiology (ESC) guidelines, with SCORE2-Diabetes applied to diabetic patients. Genetic polymorphisms in PNPLA3, TM6SF2, MBOAT7, GCKR, and HSD17B13 were analyzed in 437 patients. Finally, 104 patients underwent a 5-year follow-up to reassess muscle mass at BIA, CAP and LSM, cIMT and carotid plaques, and EFT.

Results: Patients had a mean age of 51 years, with 63% males. Patients in the lowest SMI tertile were older (54 vs 48 years, p<0.001), had lower body mass index (BMI) (27 vs 33.4 kg/m2, p<0.001), lower waist circumference (WC) (99 vs 110 cm, p<0.001), higher prevalence of dyslipidemia (56% vs 46%, p=0.03) compared to those in the highest tertile. Regarding liver damage, patients in the lowest SMI tertile had lower CAP (293 vs 317 dB/m, p<0.001), LSM (4.9 vs 6.4 kPa, p<0.001), grade 3 fibrosis prevalence (20% vs 25%, p=0.009), and MASH rates (30% vs 53%, p=0.04) compared to those in the highest tertile. For CV damage, patients in the lowest SMI tertile had a higher percentage of patients with increased cIMT (p=0.01). No significant associations were observed between SMI and the genetic polymorphisms studied. In the lowest SMI tertile, PNPLA3 CG/GG variant was linked to increased LSM (OR 1.82, p=0.03), lower cIMT (OR 0.39, p=0.04), and increased cfPWV (OR 2.1, p=0.01). The TM6SF2 wild-type allele was associated with increased cIMT (OR 3.4, p=0.004), while the CT/TT variant correlated with reduced hypertension (OR 0.09, p=0.02) and lower WC (OR 0.25, p=0.02) in the lowest SMI tertile. In multivariate analysis, low SMI was significantly associated with lower BMI (OR 0.61, p<0.001), lower WC (OR 0.92, p=0.001), female sex (OR 2.21, p=0.008), increased cIMT (OR 2.10, p=0.02), and slightly older age (OR 1.04, p=0.002). Among hypertensive MASLD patients, low SMI was independently associated with increased cIMT (OR 2.06, p=0.03) and cfPWV (OR 2.73, p=0.03). In non-diabetic MASLD patients, low muscle mass was associated with increased cIMT (OR 1.66, p=0.03), carotid plaques (OR 1.56, p=0.02), less fibrosis on histology (OR 0.29, p=0.04), lower ALT levels (OR 0.54, p=0.001), higher LDL (OR 4.38, p=0.02), and lower HDL (OR 1.03, p=0.002). At 5-year follow-up, a significant reduction in mean BMI (29.1 vs 28.4, p=0.001), a decrease in patients in the lowest SMI tertile (42% vs 22%, p<0.001), and a regression of steatosis in 9% of patients, with significant reductions in CAP (300 vs 289 dB/m, p=0.02) and LSM≥8 kPa prevalence (17% vs 7%, p<0.001) were observed. Regarding CV damage, a significant increase in carotid plaques (34% vs 54%, p<0.001), mean EFT (7.5 vs 9.0 mm, p<0.001), and high CV risk categorization (56% vs 63%, p=0.004) were observed. Baseline low muscle mass was significantly associated with changes in delta LSM (HR 2.30, p=0.013), BMI (HR 0.61, p=<0.001), and dyslipidemia (HR 5.74, p=0.007) at follow-up.

Discussion: This multicentric study revealed a significant independent relationship between sarcopenia and increased markers of atherosclerotic damage in non-cirrhotic MASLD patients, particularly among those with hypertension and without diabetes. Notably, patients with low muscle mass exhibited a lower prevalence of liver damage, possibly due to reduced visceral adiposity. However, longitudinal analysis demonstrated that low muscle mass was independently associated with an increase in mean LSM, suggesting that sarcopenia contributes to liver damage progression over time. Additionally, while persistent low muscle mass appeared to exacerbate CV damage, a definitive independent association was not observed, possibly due to the relatively short duration of follow-up. The study also identified a protective effect of the TM6SF2 polymorphism against CV damage, underscoring its potential role in mitigating CV risk.
In conclusion, this research highlights the importance of integrating imaging techniques to evaluate both liver and CV damage alongside genetic profiling for a more holistic understanding of the interplay between sarcopenia and CV damage in non-cirrhotic MASLD patients. Preserving muscle mass emerges as a key strategy for preventing both liver and CV diseases, and future public health initiatives should prioritize interventions aimed at supporting and enhancing muscle mass health.

1. INTRODUCTION

Sarcopenia was first described in 1989 by Rosenberg as the gradual loss of muscle mass over time [1]. In 2018, the European Working Group on Sarcopenia in Older People (EWGSOP) expanded upon this definition, describing sarcopenia as a progressive decline in skeletal muscle mass (SMM) characterized by the deterioration of muscle mass, strength, and physical performance [2]. Since 2016, sarcopenia has been officially recognized as a muscle disease in the International Classification of Disease (ICD-10) [3], and it has been linked to increased morbidity, mortality, low quality of life, and higher healthcare demand [4]. Although considered only an age-related condition for a long time [5], recent evidence suggested that muscle mass decline can begin as early as 40 years of age. A 2022 meta-analysis encompassing 692,056 individuals reported that the prevalence of sarcopenia in the general population aged ≥ 60 years ranges between 10% and 27%, whereas in individuals under 60 years old from 8% to 36% [6]. Projections indicate that sarcopenia prevalence will rise significantly in the coming years, becoming a major public health concern [7]. Furthermore, sarcopenia has been identified as an independent prognostic factor in several chronic diseases, such as type 2 diabetes mellitus (T2DM) [8], cardiovascular (CV) disease [9], chronic liver diseases (CLD) [10,11], and cancer [12].

Among patients with T2DM, poor glycemic control and the presence of diabetes-related complications significantly increase the risk of sarcopenia and even prediabetes has been linked to an elevated risk of sarcopenia [13]. Moreover, along with classical metabolic conditions predisposing to CV disease, as T2DM, sarcopenia has also been associated with CV diseases including myocardial infarction, valvular heart disease, chronic heart failure, atrial fibrillation, and stroke [14]. In CLD, sarcopenia has been classically linked to cirrhosis, hepatocellular carcinoma (HCC), and end-stage liver disease, and it has been correlated with increased mortality and poor prognosis also in liver transplant patients [15-17].

Sarcopenia is classified as primary when consequent to aging without other underlying causes, and as secondary when associated with chronic diseases [18]. The EWGSOP guidelines recommended dual-energy X-ray absorptiometry (DXA), computed tomography (CT), and magnetic resonance imaging (MRI) for assessing muscle mass, with MRI considered the gold standard [2]. However, due to the use of ionizing radiation and the associated costs, these methods are limited in routine clinical practice [19,20]. Bioelectrical impedance analysis (BIA), a portable and radiation-free technique, has been proven useful in assessing SMM and has been validated in large clinical trials [21,22]. Nevertheless, due to the high variability in diagnostic tools, cut-offs, and the lack of standardized reference criteria, estimating the prevalence of sarcopenia remained challenging. Moreover, gender differences have also been observed in both primary and secondary sarcopenia. In fact, men appear more susceptible to sarcopenia, while sarcopenic women experience higher mortality rates than their male counterparts [23,24]. Additionally, women over 60 years old tend to exhibit a more rapid decline in muscle strength and physical performance [25].

In 2023, an expert panel introduced the term metabolic-dysfunction associated steatotic liver disease (MASLD) to replace the term non-alcoholic fatty liver disease (NAFLD) to define patients with liver steatosis and, at least, one metabolic risk factor [26] with strict alcohol consumption (<20/30 g/day for males/females). The term MASLD is considered more inclusive than NAFLD, with the majority of NAFLD patients meeting MASLD criteria. However, MASLD patients are generally older and have a slightly higher mortality risk than NAFLD patients [27]. A minority of NAFLD patients who

do not meet MASLD criteria are classified as cryptogenic steatotic liver disease (SLD) [26]. SLD encompasses a spectrum of conditions from MASLD to metabolic-alcohol-associated liver disease (MetALD) and ALD, characterized by alcohol consumption between 20/30 g/day for women and 50/60 g/day for men and exceeding 50 g/day for women and 60 g/day for men respectively [26].

The pathogenesis of MASLD is multifactorial, involving both environmental and genetic factors. Diets rich in carbohydrates and saturated fats, along with physical inactivity, promote lipid accumulation in the liver, primarily in the form of triglycerides (TGs). This process induces lipotoxicity, mitochondrial dysfunction, and chronic inflammation through the production of proinflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin (IL)-1 and 6, and reactive oxygen species (ROS), which further exacerbate organ damage [28]. Moreover, insulin resistance (IR) accelerates de novo lipogenesis, increases free fatty acids (FFAs) production, and promotes FFAs uptake by the liver, leading to further mitochondrial dysfunction and ROS production [29]. In recent years, genome-wide association studies (GWAS) have identified genetic polymorphisms that influence the development and progression of MASLD, culminating in HCC [30,31]. The first genetic locus associated with liver steatosis, inflammation and fibrosis, was the patatin-like phospholipase domain-containing 3 (PNPLA3) single nucleotide polymorphism (SNP) [32], which encodes a protein involved in TGs hydrolysis. The I148M mutation impairs TGs hydrolysis, leading to their accumulation in the liver [33]. Another SNP associated with the development of steatosis and its progression towards advanced disease is located in the transmembrane 6 superfamily member 2 (TM6SF2) gene, which encodes a protein responsible for the secretion of very low-density lipoprotein (VLDL) from the liver. The E167K variant impairs VLDL secretion, resulting in hepatic lipid accumulation [34]. Additional SNPs, such as those in membrane bound O-acyltransferase domain containing 7 (MBOAT7) and glucokinase regulator (GCKR), have been implicated in the development and progression of liver steatosis [35,36]. Conversely, in 2018, a loss-of-function variant in 17β-hydroxysteroid dehydrogenase type 13 (HSD17B13) was associated with a reduced risk of SLD and its progression to steatohepatitis [37].

The onset and progression of liver fibrosis is a critical determinant of adverse long-term outcomes in patients with MASLD [38]. Fibrosis severity significantly impacts both liver-related and overall mortality, increasing from simple steatosis (1.2 and 0.7 per 1,000 person-years, respectively) to metabolic-associated steatohepatitis (MASH) with advanced fibrosis (5.5 and 2.5 per 1,000 person-years), rising further in cirrhotic patients (22.3 and 5.5 per 1,000 person-years), as observed in a Swedish cohort of 10,568 patients with biopsy-proven MASLD [39]. Liver fibrosis also increases the risk of liver-related complications such as hospitalization and HCC, underscoring its role as a key predictor of adverse outcomes [40].

Recognizing the prognostic importance of liver fibrosis, the 2024 European Association for the Study of Liver disease (EASL) guidelines recommend routine assessment of liver fibrosis in all MASLD patients using non-invasive tests [41]. The guidelines advocate a multistep approach, beginning with the FIB-4 score, which incorporates age, platelet count, and transaminase levels. Based on FIB-4 values, further stratification occurs: patients with FIB-4 < 1.3, should have periodic re-evaluations every 1-3 years, while those with FIB-4 > 2.67 warrant immediate Hepatology referral. For individuals with intermediate scores (1.3-2.67), additional validated testing, such as the enhanced liver fibrosis (ELF) test or liver stiffness measurement (LSM) through the vibration-controlled transient elastography (VCTE), is recommended. LSM values ≥ 8 kPa also suggest the need for Hepatology

referral, while lower values allow for primary care management with annual FIB-4 reassessment [41].

Sarcopenia and MASLD have a mutual relationship. Patients with MASLD are at an increased risk of developing sarcopenia [42] and, on the other hand, patients with sarcopenia are more likely to develop MASLD compared to those with higher muscle mass [43,44]. The first significant association between sarcopenia and NAFLD was identified in 2013 by Moon and colleagues in a cohort of Korean patients [45], and subsequent studies, primarily conducted in Asian populations, have confirmed this link [46,47]. In particular, a meta-analysis of 63,330 MASLD patients reported a prevalence of sarcopenia of around 23% in MASLD, with higher rates observed in males and Asian populations, particularly when BIA was used to assess muscle mass [48]. The same meta-analysis reported an independent association between sarcopenia and MASLD (OR 2.08) [48].

Both conditions share several common pathogenetic mechanisms, including IR, low-grade chronic systemic inflammation, oxidative stress, alterations in adipokines and myokines, and mitochondrial dysfunction. In muscle, IR promotes protein catabolism and autophagy, contributing to muscle wasting [49]. The resulting reduction in muscle mass creates a vicious cycle, exacerbating gluconeogenesis, proteolysis, and glucose intolerance [50]. In adipose tissue, IR increases lipolysis, leading to elevated levels of FFAs in the bloodstream. These FFAs accumulate in the liver, fostering hepatic fat deposition, while simultaneously contributing to myosteatosis and sarcopenia in muscle tissue [51]. Hyperinsulinemia, a consequence of IR, upregulates the sterol regulatory element-binding protein-1c (SREBP-1c), the key transcription factor involved in de novo lipogenesis, further reducing β-oxidation and promoting the accumulation of FFAs and TGs in the liver [52]. Finally, the chronic low-grade inflammation observed in MASLD enhances the breakdown of muscle proteins and impairs muscle anabolism through the overproduction of ROS and mitochondrial dysfunction, contributing to sarcopenia [14].

Muscle loss also leads to decreased levels of myokines such as irisin, which normally exert protective effects against fatty liver by enhancing hepatic insulin sensitivity and reducing inflammation [53]. The observed reduction in irisin levels in sarcopenic patients may thus contribute to the development of MASLD [51,54]. In the context of metabolic syndrome and obesity, adipocytes undergo hypertrophic and hyperplastic changes, with adipose tissue becoming infiltrated by pro-inflammatory macrophages. Inflamed adipose tissue secretes various pro-inflammatory cytokines and adipokines, including leptin, adiponectin, and myostatin. The upregulation of leptin and myostatin, along with the downregulation of adiponectin, promotes the production of ROS in the liver, which exacerbates hepatic inflammation [55]. In muscle, myostatin also inhibits myogenesis and accelerates muscle wasting while contributing to the development of IR, T2DM, and obesity [56].

Chronic low-grade systemic inflammation, a common feature of both MASLD and sarcopenia, further drives muscle protein catabolism and lipogenesis through the production of ROS mediated by TNFα and IL1 [57]. Moreover, TNFα plays a key role in sustaining the inflammatory environment by activating nuclear factor-kB (NFkB) and promoting IR, which stimulates adipose tissue to release additional adipokines [58]. ROS-induced mitochondrial dysfunction worsens the balance between protein synthesis and degradation, while impairing AMP-activating protein kinase (AMPK) signalling. AMPK dysregulation increases autophagy and mitophagy, leading to mitochondrial damage, characterized by increased mitochondrial size and reduced mitochondrial DNA [59]. Mitochondrial

dysfunction also contributes to IR by decreasing the expression of glucose transporter type 4 (GLUT4), impairing glucose uptake in muscle and liver, and perpetuating hyperinsulinemia and IR [59]. Mitochondrial dysfunction has been documented in sarcopenic patients, where it impairs muscle function, while in MASLD patients it is a key driver of liver inflammation and the progression to more advanced liver disease, as stated in a recent review carried out by our group [60].

Sarcopenia is highly prevalent in advanced CLD, including MASLD, and has been linked to a higher risk of liver fibrosis (OR 1.49) as stated in a recent meta-analysis [61]. Importantly, the negative effect of sarcopenia on liver fibrosis appears to be independent of other metabolic factors like IR and obesity [62-64]. Furthermore, a meta-analysis conducted in 2022 including 6,965 patients with cirrhosis showed that sarcopenia significantly worsens outcomes in cirrhosis, with a 2.6-fold increased mortality risk compared to patients with cirrhosis but without sarcopenia, particularly in patients with lower MELD score [65]. Sarcopenia has been demonstrated to have a role also in the setting of HCC. Indeed, it seems to adversely affect treatment response across all treatment options, such as transplantation, surgery, locoregional, and systemic treatments, as highlighted in a review conducted by our group [66]. However, two recent studies, one conducted in Asia and one Italian multi-centric study, have suggested that the deleterious effects of sarcopenia in advanced liver disease may be mediated by myosteatosis [67,68], a condition characterized by fat infiltration in muscle tissue [69]. Muscle fat content, rather than muscle mass alone, may be linked to liver disease severity, including progression to steatohepatitis and fibrosis [70]. Moreover, a 2022 study using bidirectional Mendelian randomization, did not find evidence of a causal relationship between sarcopenia and NAFLD [71], however additional longitudinal studies are needed to evaluate the impact of both sarcopenia and myosteatosis on liver damage. The prevalence and implications of sarcopenia in the early stages of MASLD have been explored more recently [5,8,9,60]. Sarcopenia appears to be directly linked to the progression of liver fibrosis in the early stages of MASLD [64,72] and is associated with increased all-cause mortality (OR 1.59), as reported in a recent meta-analysis [48]. Despite the role of sarcopenia in the prognosis of MASLD, it is not routinely evaluated in the early stages of CLD.

Beyond merely liver involvement, both MASLD and sarcopenia exert their negative influence on the cardiovascular profile. CV events represent the leading cause of mortality among patients with MASLD [73] and MASLD is now recognized as an independent risk factor for atherosclerotic CV diseases, including stroke, myocardial infarction, and carotid atherosclerosis, as well as for non-atherosclerotic conditions like cardiomyopathies, atrial fibrillation, and valvular disease [74]. The incidence of CV disease in MASLD patients is estimated at 2.69 per 1,000 person-years, with CV risk significantly amplified in the presence of metabolic comorbidities, particularly T2DM and hypertriglyceridemia [75,76]. Notably, in diabetic patients, the coexistence of MASLD increases the risk of ischemic heart disease (HR 1.18), as shown in a recent study conducted on 15,208 diabetic patients [77]. Interestingly, the degree of hepatic steatosis appears to be directly proportional to the risk of subclinical atherosclerosis, as indicated by a recent meta-analysis of 147,411 patients without a previous CV event. In this study, the risk of subclinical atherosclerosis, defined by markers such as increased carotid intima-media thickness (cIMT), arterial stiffness, or coronary artery calcium (CAC) score increased from 1.27-fold to 1.68-fold in patients with mild to moderate or severe steatosis [78].

Other key indicators of CV damage in MASLD patients include arterial stiffness and epicardial fat thickness (EFT).

Arterial stiffness, an early marker of atherosclerotic changes and cardiac hypertrophy, is often increased in MASLD patients [79]. A prospective study using data from the NHANES cohort, with a follow-up period of 26.3 years, demonstrated that a 1 m/s increase in pulse wave velocity (PWV), a measure of arterial stiffness, was associated with a 44% increase in overall mortality and a 53% increase in CV mortality in MASLD patients, irrespective of confounding factors [80]. Interestingly, in patients who experienced regression of steatosis, PWV showed a significantly smaller annual increase (1.27 cm/s/year) compared to those with persistent steatosis (6.75 cm/s/year), highlighting the potential reversibility of early vascular changes with disease improvement [81]. Nevertheless, the relationship between PWV and liver fibrosis remains a topic of debate. While some studies have found no significant correlation between PWV and histological fibrosis degree [82,83], a longitudinal study suggests that PWV increases over time in parallel with liver fibrosis, as measured by LSM [84].

EFT refers to the presence of visceral fat between the heart and the pericardium, and it is recognized as an indicator of CV risk. An increase in EFT is associated with coronary artery disease, left ventricular dysfunction, and atrial fibrillation [85]. EFT can be non-invasively assessed using either MRI or transthoracic echocardiography [85]. In patients with MASLD, EFT is directly correlated with the degree of liver fibrosis, with more advanced fibrosis linked to greater EFT [86,87]. Moreover, EFT is independently associated with CV damage, manifesting as increased cIMT and carotid plaques, regardless of other metabolic risk factors [88]. These associations between EFT, liver steatosis, fibrosis, and CV damage were further corroborated in a meta-analysis involving 2,260 patients, including both MASLD and control subjects [89].

Among MASLD features, the primary determinant of CV damage appears to be the degree of liver fibrosis [90]. In fact, histological fibrosis is strongly associated with increased cIMT and carotid plaques, making it a critical marker for assessing CV risk [91]. However, subclinical atherosclerosis itself seems to develop independently of fibrosis severity [92].

MASLD is characterized by a complex interplay between metabolic dysregulation and systemic inflammation, thus predisposing to CV disease. A central role is kept by IR as it reduces high-density lipoprotein (HDL) and increases levels of small-dense low-density lipoprotein (sdLDL), remnants lipoprotein, and VLDL, which collectively foster a proatherogenic environment [93]. Low-grade systemic chronic inflammation, commonly observed in MASLD, is closely associated with increased CV morbidity and mortality [94]. In MASLD, endothelial dysfunction is driven by the overexpression of selectin-E, vascular cell adhesion molecule-1 (VCAM-1), and intercellular cell adhesion molecule-1 (ICAM-1), fostering the development of atherosclerotic plaque [95]. In particular, endothelial dysfunction recalls peripheral monocytes that once in the arterial intima differentiate into macrophages, able to capture oxidized LDL and TGs-rich lipoprotein remnants evolving into foam cells, which contribute to the creation of fatty streaks in the arterial wall [95]. Additionally, intima macrophages in MASLD overexpress scavenger receptors, further accelerating atherosclerotic plaque formation by increasing lipoprotein intake [96]. The proinflammatory phenotype of these macrophages (M1 macrophages) exacerbates the production of proinflammatory cytokines, thereby perpetuating the chronic systemic inflammation that characterizes both MASLD and CV disease [95]. The persistent inflammation not only accelerates the development of atherosclerosis but also leads to endothelial dysfunction, further contributing to CV risk [95].

Also sarcopenia is strictly linked with CV disease, such as ischemic heart disease, heart failure, coronary artery disease, and atrial fibrillation [97]. In patients with CV disease, sarcopenia is more prevalent than in the general population, ranging from 31% to 39%, with a slightly higher prevalence in men (35%) compared to women (32%), as stated in a meta-analysis involving 4,327 patients [97]. Sarcopenia, particularly when combined with obesity, a condition known as sarcopenic obesity, has been linked to an increased risk of CV disease, including heart failure and stroke, as reported in a Chinese longitudinal study [98].

Sarcopenia is associated also with subclinical atherosclerosis. In fact, a reduction in muscle mass is associated with an increase in both cIMT [99], and carotid plaques, particularly in patients with T2DM [100], despite the association between sarcopenia and carotid plaques in non-diabetic patients remains less defined.

Interestingly, the relationship between sarcopenia and CV disease appears to be unidirectional, with the loss of muscle mass predisposing to CV damage. However, the presence of major CV events, such as stroke, myocardial infarction, or heart failure, does not seem to exacerbate the occurrence of sarcopenia, whether measured by muscle mass or strength [101].

Sarcopenia and CV disease share common pathogenetic pathways, including chronic low-grade inflammation, mitochondrial dysfunction, and IR [14]. The resulting increase in ROS triggers apoptosis and dysfunction in muscle cells, leading to enhanced protein catabolism and loss of muscle mass [14]. In patients with both CV disease and sarcopenia, endothelial dysfunction plays a crucial pathogenetic role [14]. Chronic inflammation reduces insulin-like growth factor-1 (IGF-1), which is associated with plaque stability in murine models [102]. Additionally, proinflammatory cytokines impair endothelial plasticity and reactivity, diminishing muscle perfusion and reducing the uptake of oxygen and essential amino acids. This mechanism impairs protein synthesis, contributing to further muscle wasting [103]. Age-related increases in chronic inflammation, a process known as inflammaging [104], may further exacerbate these changes, compounding the effects of both sarcopenia and CV disease. Nevertheless, further studies are needed to clarify the full extent of these interactions.

Even though MASLD, sarcopenia, and CV disease share common pathogenetic pathways, relatively few studies have specifically investigated the interplay between sarcopenia and both CV and liver damage in MASLD patients. A Korean study involving 7,248 patients with hepatic steatosis demonstrated that the risk of sarcopenia (OR 2.71) and CV risk (OR 2.79) was significantly elevated in MASLD patients, regardless of liver fibrosis, particularly among patients with lower metabolic risk profiles [105]. Another Korean study from 2020 found that patients with MASLD and sarcopenia had a higher CV risk (OR 1.83), and the presence of sarcopenia, when combined with advanced liver fibrosis, further increased the CV risk (OR 3.56) compared to those without sarcopenia or isolated fibrosis [106]. Additionally, MASLD patients with sarcopenia showed an elevated risk of both all-cause mortality (1.69-fold) and CV mortality (2.17-fold). Notably, MASLD patients without sarcopenia showed overall and CV mortality rates comparable to the general population, suggesting the key role of sarcopenia in driving mortality among MASLD patients [107]. Finally, a large-scale Greek study further reinforced these findings, showing that patients with liver steatosis, as assessed by the hepatic steatosis index (HSI), combined with low skeletal muscle mass and central obesity, had a significantly increased risk of CV event over a 10-years follow-up period [108]. Another study conducted in 2021 assessed CV damage in MASLD patients by directly measuring the cIMT and the

presence of carotid plaque, and the authors reported that low muscle mass was independently associated with increased cIMT (OR 3.3) and carotid plaques (OR 3.54) in obese MASLD patients [109].

Despite this background, the majority of studies exploring the association between sarcopenia, liver, and CV damage in MASLD patients have important limitations, and the precise mechanisms linking these conditions remain poorly understood.

First, most of these studies are cross-sectional and predominantly focus on Asian populations. As known, ethnicity significantly influences body composition, with Asian populations tending to exhibit higher degrees of adiposity at lower body mass index (BMI) compared to non-Asians. For this reason, in Asiatic countries lower cut-offs of BMI are used to define overweight and obesity [110]. This ethnic variation in body composition complicates the generalizability of findings across different populations highlighting the need for more inclusive research. Secondly, the paucity of longitudinal studies makes it difficult to determine whether the relationship between sarcopenia and CV or liver damage is bidirectional, or if sarcopenia is solely a consequence of metabolic and inflammatory disfunction in MASLD. Furthermore, sarcopenia is often assessed using the skeletal muscle index (SMI), which has several limitations. Several studies have normalized SMI by BMI or body weight. However, in MASLD, a considerable proportion of patients also experience obesity, which can introduce a significative bias. Normalizing for height instead of weight may reduce such bias, but no specific validated SMI cut-off exists for defining sarcopenia in MASLD patients.

Moreover, many studies examining the impact of sarcopenia on CV damage in MASLD focus on CV risk scores rather than directly assessing CV damage, potentially overestimating the true impact of sarcopenia on CV damage. Similarly, liver steatosis and fibrosis are often assessed using non-invasive scoring systems rather than imaging techniques or histological analysis, which may limit the accuracy of the results.

Finally, no studies to date have explored the potential contribution of genetic variants predisposing to liver damage in the risk of developing sarcopenia and CV damage. Important confounding factors such as lifestyle, including nutrition and physical activity, are also frequently underexamined in these studies, limiting the ability to fully understand the complex interactions between MASLD, sarcopenia, and CV disease.

As the prevalence of MASLD, sarcopenia, and CV diseases continues to rise globally [111-113], understanding their complex interactions has become a critical public health issue. Gaining insight into the relationship between sarcopenia, MASLD, and CV damage is essential for improving patient management. Clarifying these links will help develop better diagnostic tools and risk assessment models, allowing clinicians to identify high-risk patients more accurately. This could improve early treatment focused on preserving muscle mass through exercise and nutrition, potentially reducing the incidence of CV events and liver disease progression and improving outcomes for MASLD patients. In turn, this would reduce healthcare costs and improve long-term patient quality of life. Furthermore, recognizing ethnic and genetic differences in sarcopenia predisposition, as well as in severe liver and CV damage, ensures that interventions are applicable across different populations, promoting greater equity in healthcare.

Given the limited number of comprehensive studies addressing the interaction between MASLD, sarcopenia, and CV damage, this project aims to contribute to the field by providing a thorough

investigation into the combined effects of sarcopenia and MASLD on CV damage, using imaging techniques and genetic analysis to elucidate the underlying mechanisms in a cohort of non-cirrhotic MASLD patients. The specific objectives of this research are:

1. To assess the influence of low muscle mass, measured using a non-invasive and radiation-free technique as BIA, on both liver and CV damage within a cohort of non-cirrhotic MASLD patients.
2. To investigate the impact of genetic variants associated with liver damage on muscle mass and CV damage, identifying whether genetic predisposition exacerbates or influences these conditions in the same patient cohort.
3. To track longitudinal changes in metabolic, liver, muscle mass, and CV parameters over a 5-year follow-up period, examining how baseline muscle mass and its changes affect liver and CV disease progression in a subset of these patients.

2. MATERIALS AND METHODS

2.1 Study design and population

We conducted a multicentric cross-sectional study involving 856 non-cirrhotic MASLD patients. Of these, 526 were recruited from the outpatient hepatology clinics of Medicine and Metabolic Liver Disease Unit at Policlinico Hospital of Milan (Milan, Italy), 190 from the Gastrointestinal and Liver Unit at Palermo University Hospital (Palermo, Italy), and 140 from the Liver Unit at Imperial College Healthcare NHS Trust (London, United Kingdom).

Inclusion criteria were age greater than 18 years old, abdominal ultrasound (US) proven MASLD, and the capacity to provide informed consent. Exclusion criteria were the presence, suspected by clinical, laboratory, or histological data of liver cirrhosis, pregnancy, patients with known muscular degenerative disease, congestive heart failure, ascites, active neoplasia, and patients with life expectancy lower than two years, in order to avoid muscle function impairment due to secondary causes.

The study protocol was approved by the Institutional Ethics Committee of Milan (N-0004629-U). All patients provided written informed consent to participate in the study according to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2 Clinical and laboratory data

The medical history, smoke habits, alcohol consumption, expressed as an average daily grams of alcohol, physical activity, and current therapy were collected at enrollment, for all patients. Alcohol consumption lower than 30/20 gr/die for men and women, respectively, defined a low alcohol consumption, alcohol consumption between 30 and 60 gr/die for men and between 20 and 50 gr/die for women defined a moderate alcohol consumption or MetALD [26].

We also recorded anthropometric data such as weight, height, and waist circumference (WC). A BMI of 25-29.9 kg/m2 defined overweight, whereas BMI ≥ 30 kg/m2 defined obesity. As WC thresholds, we considered both stricter (WC > 94/80 cm in men/women, respectively) and higher criteria (WC > 102/88 cm in men/women, respectively) to define increased WC [26].

According to the European Society of Cardiology (ESC) guidelines 2021, physical activity was divided into aerobic and anaerobic, and patients were further categorized as inactive or active based on the intensity of physical activity [114].

Among laboratory data, for all patients glucidic and lipidic profile was performed, as well as liver enzymes and creatinine levels to evaluate kidney function. In a subgroup of 516 patients, also ferritin levels were detected. A homeostatic model assessment for insulin resistance (HOMA-IR) greater than 2.5 suggested IR and was calculated only in patients without T2DM at enrollment. Low-density lipoprotein (LDL) was calculated with the Friedewald formula. Aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) greater than 39 U/L and 41 U/L, respectively, were considered increased, such as gamma-glutamyltransferase (GGT) greater than 61/36 U/L for men and women, respectively, according to local laboratory cut-offs. Ferritin levels greater than 400 ng/mL and 150 ng/mL for men and women, respectively, were considered elevated.

2.3 Assessment of liver disease

For all patients, the presence of liver steatosis was assessed through abdominal US performed by experienced sonographers using a 3.5 MHz convex-array probe. Based on hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring liver steatosis was classified as mild (grade 1), moderate (grade 2), and severe (grade 3) [115].

The presence of liver fibrosis was non-invasively evaluated through the LSM by VCTE at Fibroscan (Echosens, Paris, France), using the M probe, or in case of unsuccessful measurement, the XL one. LSM ≥ 8 kPa suggested advanced liver fibrosis, according to international guidelines [41]. Using Fibroscan, through the controlled attenuation parameter (CAP) liver steatosis was quantified. CAP ≥ 248 dB/m and < 260 dB/m suggested the presence of mild steatosis, CAP between 260 and 280 dB/m suggested moderate steatosis, whereas CAP > 280 dB/m suggested severe steatosis.

In a subgroup of 370 patients, a percutaneous liver biopsy using US guidance was performed according to good clinical practice by a trained physician through an 18 Gauge needle. The liver sample was embedded in paraffin block and stained with hematoxylin and eosin and Masson's trichrome stains to detect fibrosis. All liver biopsies were evaluated by experienced pathologists for each center. The liver biopsies were scored using the NAFLD activity score (NAS) system and, according to the score, hepatic steatosis and lobular inflammation were scored from 0 to 3, ballooning was scored from 0 to 2, while fibrosis was scored from 0 to 4 [116].

2.4 Assessment of sarcopenia

For all patients enrolled, sarcopenia was evaluated by the measure of muscle mass through the BIA, a validated, non-invasive, and radiation-free tool, and the exam was performed after six hours of fasting. Through the passage of a low voltage alternating electrical current in the whole body, BIA provided the measure of both resistance (R) and reactance (Xc). Through R, Xc and the phase angle, the machine obtained the measure of free fat mass (FFM), fat mass (FAT), SMM, and total body water (TBW). The SMI, a validated marker of sarcopenia, was calculated as follows: SMM (kg)/height(m)^2 [21]. Due to the lack of validated cut-offs for the skeletal muscle index in patients with MASLD, we divided the SMI into tertiles as lowest, middle and highest, using gender specific cut-offs. The first tertile represented the lowest SMI, the second tertile the middle, and the third tertile the highest SMI.

Whereas in Milan (Bioimpedance Human im Plus II, DS Medica Dietosystem) and Palermo (single-frequency ElectroFluidGraph+ AKERN) cohorts the BIA machines provided directly the measure of SMM, in London cohort the BIA machine provided solely the measure of FAT and FFM. We further

normalized the FFM for height squared (FFM/height^2) and divided it into three gender-specific tertiles. As for SMI, the first tertile corresponded to the lowest FFM, the second to the middle, and the third to the highest FFM.

2.5 Assessment of CV damage

The presence of past CV events, increased cIMT or carotid plaques, or elevated cfPWV defined CV damage.

In addition, according to the 2021 ESC guidelines, the CV risk for each patient was assessed using the SCORE2 and SCORE-OP calculators. These tools take into account age, gender, smoking habits, systolic blood pressure, non-HDL cholesterol, and geographic region. CV risk was classified into low, high, and very high categories for predicting the likelihood of a major CV event within 10 years [114]. For patients with T2DM who have not experienced a previous CV event or severe organ damage, the SCORE2-Diabets calculator was used, incorporating additional diabetes-specific factors such as age at T2DM onset, glycated hemoglobin levels, and glomerular filtration rate [117].

History of past CV events, such as myocardial infarction, ischemic or hemorrhagic stroke, and previous carotid endarterectomy was recorded in all patients.

Only in the Milan and Palermo cohorts, a carotid doppler US with a 7.5 MHz transducer (Esaote MyLabX8) was performed to measure mean cIMT and detect the presence of carotid plaques. A cIMT ≥ 0.9 mm defined subclinical atherosclerosis, while carotid wall thickening > 1.2 mm defined the presence of carotid plaque [118].

Finally, only in the Milan cohort data on arterial stiffness and EFT were available.

In particular, to evaluate aortic stiffness, carotid-femoral pulse wave velocity (cfPWV) was assessed using tonometry (SphygmoCorXCEL®, AtCor Medical). Peripheral blood pressure was measured at the right brachial artery, followed by recording the distances from the carotid artery to the sternal notch and from the sternal notch to the top edge of the femoral cuff. The femoral cuff was then inflated to capture the femoral waveform, while the carotid signal was measured using a tonometer. After 10 seconds of simultaneous recording of the carotid tonometer and femoral cuff signal, the software calculated the cfPWV based on the time delay between the carotid and femoral pulse waves (pulse wave transit time) [119]. A cfPWV > 10 m/sec indicated increased vascular stiffness [119].

Additionally, transthoracic echocardiography with a 1-5 MHz transducer was performed (Philips Affiniti 70G) on all patients to measure EFT. An EFT ≥ 9.5 mm in men and ≥ 7.5 mm in women was considered a marker of visceral adiposity, whereas an EFT ≥ 5.2 mm was associated with coronary artery disease (CAD) [120].

2.6 Genetic polymorphisms

In 437 patients from the Milan and Palermo cohorts, an additional blood sample was collected, and DNA was extracted by the phenol-chloroform method. The patients were genotyped in duplicate using Taqman 5'-nuclease assay for the following single nucleotide polymorphism (SNP): rs738409 C>G (PNPLA3 I148M), rs58542926 C>T (TM6SF2 E167K), rs641738 C>T (MBOAT7 G17E), rs1260326 C>T (GCKR P446L), and rs72613567 T>A (HSD17B13) (Life Technologies, Carlsbad, CA). Genotyping success rate was > 98%.

2.7 Follow-up evaluation

In a subgroup of 104 patients from the Milan cohort, a 5-year follow-up assessment was conducted. During the re-evaluation, comprehensive medical histories were updated, including smoking and alcohol consumption habits. Additionally, any new diagnoses of hypertension, T2DM, and dyslipidemia were recorded. All patients underwent BIA to assess changes in muscle mass. Furthermore, liver damage was reevaluated using abdominal US and Fibroscan, while cIMT and carotid plaque presence were reassessed through carotid Doppler US in all patients. Finally, echocardiography was performed to reassess EFT.

2.8 Statistical analysis

Categorical variables are presented as absolute and relative frequencies (n, %), while continuous variables are expressed as mean ± standard deviation (SD) or median [interquartile range – IQR], as appropriate. Differences between categorical variables were assessed using the chi-squared test, while continuous variables were compared using either the unpaired Student's t-test for normally distributed data or the Mann-Whitney test for non-normally distributed data. To identify factors associated with the lowest SMI tertile, multivariable logistic regression analyses were performed after adjusting for major confounding factors.

Changes in clinical, laboratory, and instrumental data from baseline to five-year follow-up were analysed using paired sample t-test and Wilcoxon signed ranks test for normally and not normally distributed variables, respectively. To determine predictive factors associated with the progression of sarcopenia, liver, and CV damage during follow-up, a Cox proportional hazard regression model was employed.

A two-tailed p-value <0.05 was considered statistically significant. All statistical analyses were conducted using JMP Pro 17.2.0 (SAS, Cary, NC, USA).

3. Results

We enrolled 947 patients with SLD between 2017 and 2023. According to the definition proposed in 2023 [17], 911 fulfilled the diagnostic criteria of MASLD. 55 (6%) patients were diagnosed as cirrhotic and were excluded from further analysis. 856 patients were finally included in the analysis. In Figure 1, we provide an overview of the patients enrolled in the study.

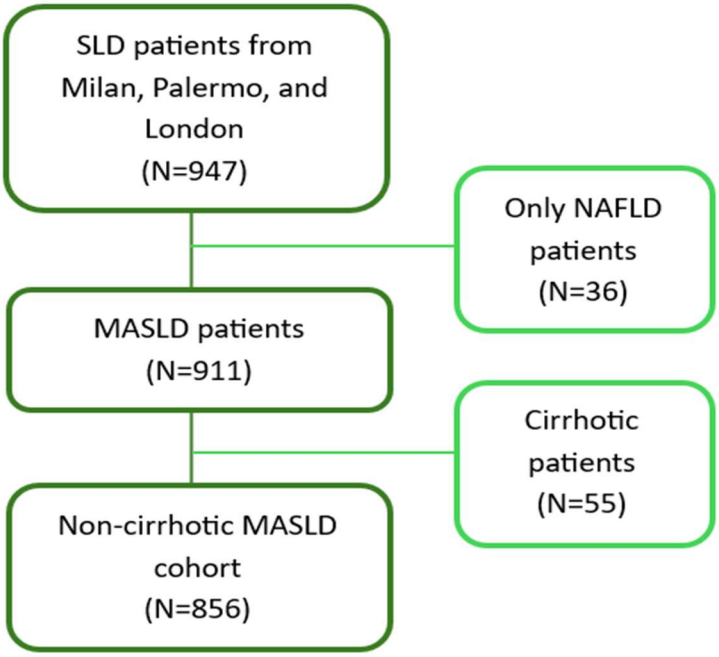

Figure 1. Diagram showing patients enrolled in the study.

The mean age of the whole cohort was 51 ± 12 years, whereas the majority of the cohort (63%) were males. 70% of the females enrolled were in the post-menopausal phase. The majority of patients were abstainers or had a low alcohol consumption, whereas 18% were current smokers. As expected in MASLD patients, almost the totality of patients was overweight (46%) or obese (43%) and had increased WC according to gender cut-offs (89% using stricter criteria, 65% using higher threshold). Most of the half of the cohort was inactive (57%). As for metabolic comorbidities, half of the cohort was dyslipidemic, 42% hypertensive, and 25% diabetic. Mean fasting glucose was 100 mg/dL, whereas 69% of patients without T2DM had insulin resistance. 50% and 25% of patients had increased ALT and AST, respectively, while 35% had increased GGT. 33% of the MASLD patients had increased ferritin levels, according to gender cut-offs. Mean triglycerides was 133 mg/dL, mean LDL was 116 mg/dL.

Among liver features, the majority of patients (67%) experienced a moderate or severe steatosis degree at US, with a mean CAP of 304 ± 53 dB/m, and 70% showed severe steatosis on Fibroscan. The mean LSM was 5.6 kPa [4.4-7.8 kPa], with 24% of the cohort exhibiting an LSM ≥ 8 kPa. Among patients with available liver histology, 52% had no or mild fibrosis, 24% had grade 2 fibrosis, and 24% had grade 3 fibrosis. Additionally, 44% of the patients had histologically documented MASH.

Among CV features, only 4% reported a previous major CV event. Conversely, more than half of the cohort had a high CV risk (59%) according to the ESC 2021 guidelines. 29% of patients had low CV risk, whereas 12% was defined as very high CV risk. Among 690 patients in which carotid US was available, 22% had increased cIMT, and 34% showed carotid plaques. Finally, among the 502 patients who underwent both echocardiography and tonometry, the mean EFT was 7.5 ± 2.5 mm. Notably, 30% of patients had an EFT ≥9.5/7.5 mm in men/women, and 83% had an EFT≥5.2 mm. Additionally, 10% of patients exhibited an increased cfPWV.

Tables 1a and 1b summarize the gender-specific cut-offs for SMI and FFM/height^2, defining the three muscle mass tertiles for the Milan and Palermo cohort, and the London cohort, respectively.

Table 1a. Tertile of SMI according to gender in the Milan and Palermo cohorts.

	Male	Female
Lowest tertile	SMI < 10.35	SMI < 7.75
Middle tertile	10.35 ≤ SMI < 11.20	7.75 ≤ SMI < 8.50
Highest tertile	SMI ≥ 11.20	SMI ≥ 8.50

SMI: skeletal muscle index.

Table 1b. Tertile of FFM/ height^2 according to gender in the London cohort.

	Male	Female
Lowest tertile	FFM/ height^2 < 19.70	FFM/ height^2 < 17.40
Middle tertile	19.70 ≤ FFM/ height^2 < 21.07	17.40 ≤ FFM/ height^2 < 19.20
Highest tertile	FFM/ height^2 ≥ 21.07	FFM/ height^2 ≥ 19.20

FFM: free fat mass.

3.1 Clinical and laboratory characteristics by SMI tertiles

Patients in the lowest tertile of SMI were older (54 vs 51 vs 48 years, p<0.001), had lower BMI (27 vs 29.7 vs 33.4 kg/m2, p<0.001), a lower percentage of obese patients (15% vs 42% vs 72%, p<0.001) and lower WC (99 vs 104 vs 110 cm, p<0.001) with a higher percentage of overweight (61% vs 51% vs 25%, p<0.001) compared to patients in the middle and higher tertile. Patients in the lowest and middle tertile, compared to the highest, had a lower percentage of T2DM (21% vs 32%, p=0.003). Conversely, patients in the lowest tertile had a higher prevalence of dyslipidemia compared to patients in the highest tertile (56% vs 46%, p=0.03). No differences between the tertiles of MASLD patients were observed regarding gender, alcohol consumption, smoking habits, physical activity, prevalence of hypertension, and previous CV events.

Regarding laboratory data, patients in the lowest tertile had a lower percentage of increased HOMA-IR (57% vs 69% vs 80%, p<0.001), increased ALT (39% vs 53% vs 58%, p<0.001), and AST (21% vs 27% vs 28%, p=0.04) compared to the other tertiles. Conversely, they showed higher LDL (122 vs 115 vs 112 mg/dL, p=0.04) and lower HDL cholesterol (45 vs 47 vs 50 mg/dL, p<0.001). No significant differences in mean glucose, GGT, ferritin, and triglycerides were noted between the tertiles.

3.2 Liver damage by SMI tertiles

MASLD patients in the lowest tertile appeared to exhibit lower severity of steatosis compared to those in the middle and highest tertiles (steatosis third degree 17% vs 27% vs 31%, p=0.001; mean CAP 293 vs 305 vs 317 dB/m, p<0.001). Regarding liver fibrosis, patients in the lowest SMI tertile had both a lower LSM compared to those in the middle (4.9 vs 5.6 kPa, p<0.001) and highest tertiles (4.9 vs 6.4 kPa, p<0.001) and a lower fibrosis degree on histology compared to those in the highest tertile (p=0.009). Additionally, patients in the lowest tertile had a lower percentage of MASH compared to those in the highest tertile (30% vs 53%, p=0.04).

Interestingly, when we considered patients with and without obesity, the association between lower LSM and lower SMI tertile was significant only in patients without obesity (mean SM in low tertile vs middle vs high 4.8 vs 5.2 vs 5.6, p=0.004) but not in patients with obesity (p=0.37).

3.3 CV damage by SMI tertiles

Patients with MASLD in the lowest tertile of SMI had a significantly higher incidence of increased cIMT (p=0.01) and EFT≥5.2 mm compared to patients in the middle tertile (85% vs 77%, p=0.05). However, no significant differences were observed in carotid plaques (p=0.12), mean EFT (p=0.11), CV risk (p=0.35), or increased cfPWV (0.13), but a trend of increasing carotid plaques and cfPWV was

noted across the SMI tertiles, with the higher increase in carotid plaques and cfPWV occurring in the lowest SMI tertile.

The clinical characteristics of the MASLD cohort based on SMI tertiles were summarized in Table 2a.

Table 2a. Clinical, laboratory, liver, and CV features of MASLD patients classified by tertiles of SMI.

Variables	MASLD lowest tertile (N=287)	MASLD middle tertile (N=283)	MASLD highest tertile (N=287)	Overall p value	Highest vs Lowest	Highest vs Middle	Middle vs Lowest
Demographic and Anthropometric features							
Age, years	54±12	51±12	48±13	**<0.001**	**<0.001**	**0.005**	**0.006**
Alcohol consumption, gr/day >Abstainers, N (%) >Low, N (%) >MetALD, N (%)	 161 (56) 106 (37) 20 (7)	 170 (60) 99 (35) 14 (5)	 161 (56) 100 (35) 26 (9)	0.76	0.91	0.39	0.63
Active smokers, N (%)	49 (17)	42 (15)	63 (22)	0.20	0.42	0.15	0.25
Mean BMI, kg/m2 >Overweight, N (%) >Obesity, N (%)	27.0±3.3 175 (61) 42 (15)	29.7±3.8 143 (51) 118 (42)	33.4±5.1 73 (25) 208 (72)	**<0.001** **<0.001** **<0.001**	**<0.001** **<0.001** **<0.001**	**<0.001** **<0.001** **<0.001**	**<0.001** **0.01** **<0.001**
Mean WC, cm >WC>94/80 cm in M/W, N (%) >WC>102/88 cm in M/W, N (%)	99±10 247 (86) 155 (54)	104±10 252 (89) 184 (65)	110±13 270 (94) 221 (77)	**<0.001** **0.02** **<0.001**	**<0.001** **0.006** **<0.001**	**<0.001** *0.05* **0.002**	**<0.001** 0.52 **0.009**
Physical activity >Inactive, N (%) >Regular activity, N (%)	 161 (56) 126 (44)	 153 (54) 130 (46)	 115 (40) 172 (60)	0.48	0.45	0.31	0.76
Metabolic Comorbidities							
T2DM, N (%)	61 (21)	62 (22)	93 (32)	**0.003**	**0.003**	**0.006**	0.92
Hypertension, N (%)	121 (42)	113 (40)	125 (44)	0.68	0.80	0.40	0.61
Dyslipidemia, N (%)	161 (56)	130 (46)	132 (46)	**0.03**	**0.03**	0.93	**0.02**
Laboratory data							
Fasting glucose, mg/dL	100±17	98±21	103±28	0.10	0.16	**0.02**	0.31
HOMA-IR >2.5, N (%)*	90 (57)	119 (69)	133 (80)	**<0.001**	**<0.001**	**0.03**	**0.02**
ALT, UI/L >Increased ALT, N (%)	36[23-57] 112 (39)	43[29-65] 150 (53)	46[31-73] 166 (58)	**<0.001** **<0.001**	**<0.001** **<0.001**	0.23 0.25	**0.0002** **0.002**
AST, UI/L >Increased AST, N (%)	27[21-36] 60 (21)	30[24-41] 76 (27)	31[23-41] 80 (28)	**0.001** 0.09	**0.002** **0.04**	0.78 0.77	**0.002** 0.09
GGT, UI/L >Increased GGT, N (%)	34[22-67] 98 (34)	41[24-83] 116 (41)	36 [25-67] 92 (32)	0.14 0.10	0.31 0.63	0.33 *0.05*	*0.05* 0.14
Ferritin**, ng/mL >Increased ferritin, N (%)	213[97-415] 66 (37)	207[88-394] 60 (33)	179[82-329] 43 (28)	0.68 0.18	0.36 0.08	0.58 0.29	0.82 0.44
Triglycerides, mg/dL	136[95-179]	126[93-178]	136[98-184]	0.25	0.27	0.11	0.61
LDL, mg/dL	122±40	115±42	112±39	**0.04**	**0.01**	0.36	0.09

HDL, mg/dL	45±14	47±13	50±12	**<0.001**	**<0.001**	0.07	**0.01**
Liver Features							
US steatosis degree				**0.001**	**0.0001**	0.33	**0.009**
>1, N (%)	112 (39)	92 (32)	78 (27)				
>2, N (%)	125 (44)	115 (41)	119 (41)				
>3, N (%)	50 (17)	76 (27)	90 (31)				
CAP, dB/m	293±54	305±51	317±49	**<0.001**	**<0.001**	**0.03**	**0.02**
CAP degree							
>Mild, N (%)	11 (5)	12 (6)	6 (3)	0.49	0.46	0.33	0.83
>Moderate, N (%)	26 (12)	20 (10)	16 (9)	0.61	0.41	0.86	0.54
>Severe, N (%)	145 (63)	146 (74)	142 (79)	**0.02**	**0.007**	0.17	0.22
LSM, kPa	4.9[4-6.7]	5.6[4.5-7.8]	6.4[4.9-9.0]	**<0.001**	**<0.001**	**0.002**	**<0.001**
>LSM≥8kPa, N (%)	43 (15)	68 (24)	95 (33)	**<0.001**	**<0.001**	**0.02**	**0.02**
Fibrosis at histology				0.07	**0.009**	0.43	0.13
>MASH, N (%)	16 (30)	31 (44)	39 (53)	**0.04**	**0.02**	0.32	0.14
>Fibrosis 0-1, N (%)	47 (63)	52 (52)	57 (46)				
>Fibrosis 2, N (%)	13 (17)	22 (22)	37 (29)				
>Fibrosis 3, N (%)	15 (20)	27 (26)	32 (25)				
CV Features							
cIMT≥0.9 mm, N (%)***	58 (28)	27 (15)	33 (23)	**0.01**	0.27	0.11	**0.003**
Carotid plaques, N (%)***	94 (39)	73 (32)	70 (32)	0.12	0.14	0.92	0.18
Mean cfPWV, m/sec	7.8±1.9	7.7±1.7	7.6±2.1	0.39	0.19	0.48	0.46
>Increased cfPWV, N (%)****	43 (14)	30 (7)	29 (8)	0.13	0.18	0.81	0.09
Mean EFT, mm****	7.5±2.4	7.3±2.6	7.8±2.3	0.11	0.19	0.10	0.67
>EFT≥9.5/7.5 mm in M/W, N (%)	55 (28)	49 (29)	47 (33)	0.60	0.34	0.46	0.91
>EFT≥5.2 mm, N(%)	165 (85)	129 (77)	121 (86)	0.08	0.88	*0.06*	*0.05*
CV risk categories				0.35	0.21	0.42	0.37
>Low, N (%)	75 (26)	87 (31)	93 (32)				
>High, N (%)	178 (62)	168 (59)	156 (55)				
>Very high, N (%)	34 (12)	28 (10)	38 (13)				
Previous CV events, N (%)	12 (4)	14 (5)	9 (3)	0.36	0.42	0.26	0.38

*Data available in 498 patients. **Data available in 516 patients. ***Data available in 690 patients. ****Data available in 502 patients.

Numbers in bold represent statistical significance.

Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; CAP: controlled attenuation parameter; cfPWV: carotid-femoral pulse wave velocity; cIMT: carotid intima-media thickness; CV: cardiovascular; EFT: epicardial fat thickness; GGT: gamma-glutamyl transferase; HDL: high density lipoprotein; HOMA-IR: homeostatic model assessment for insulin resistance; LDL: low density lipoprotein; LSM: liver stiffness measurement; MASH: metabolic-dysfunction associated steatohepatitis; MASLD: metabolic-dysfunction associated steatotic liver disease; MetALD: metabolic alcohol-associated liver disease; T2DM: type 2 diabetes mellitus; US: ultrasound; WC: waist circumference.

3.4 Genetic polymorphisms in the whole cohort and by SMI tertiles

Genetics and liver and CV damage in the whole cohort.

As expected, in the whole cohort, patients with increased LSM, compared to others, showed a higher prevalence of the PNPLA3 CG/GG (67% vs 56%, p=0.04) and the MBOAT7 CT/TT (19% vs 17%, p=0.04) genotypes. Conversely, no significant differences were detected for the other polymorphisms studied, even when fibrosis degree was evaluated at histology, probably due to the smaller number of patients with advanced liver fibrosis.

Moreover, patients with the PNPLA3 CG/GG polymorphism exhibited an increase in cfPWV (88% vs 58%, p=0.01), whereas those with TM6SF2 CT/TT polymorphisms had a lower percentage of increased cIMT (2% vs 22%, p=0.0002) but a higher prevalence of EFT ≥ 5.2 mm (21% vs 9%, p=0.04) compared to the wild type. Additionally, patients with the GCKR CT/TT and the HSD17B13 TTA/TATA polymorphism were found to have a higher CV risk compared to others (GCKR 96% vs 79%, p=0.02; HSD17B13 52% vs 31%, p=0.03). The MBOAT7 variant was not associated with markers of CV damage.

The PNPLA3 GG and the HSD17B13 TT variants were more prevalent in female MASLD patients (PNPLA3 29% vs 15%, p=0.0003; HSD17B13 76% vs 64%, p=0.01).

No other significant differences in liver and CV damage, metabolic comorbidities, or laboratory data were observed among the genetic polymorphisms analyzed.

Genetics and liver and CV damage according to SMI tertiles.

In the 437 patients whose genetic polymorphisms were evaluated, no significant associations were found between SMI tertiles and the polymorphisms in PNPLA3 (p=0.06), TM6SF2 (p=0.20), MBOAT7 (p=0.66), GCKR (p=0.54), or HSD17B13 (p=0.37).

To increase statistical power, further analysis combined patients in the low and middle SMI tertiles into a single category for comparison with those in the highest SMI tertile.

Regarding liver damage, the PNPLA3 genetic variant was associated with increased LSM on Fibroscan (OR 1.82, 95% CI 1.1-3.1, p=0.03) in patients with the highest SMI tertile. No significant differences were found in the TM6SF2, MBOAT7, GCKR, and HSD17B13 polymorphisms between the lowest and highest SMI tertile concerning liver fibrosis.

As for CV damage, the PNPLA3 CG/GG variant was associated with lower cIMT (OR 0.39, 95% CI 0.15-0.96, p=0.04) in the lowest SMI tertile and with increased cfPWV (OR 2.1, 95% CI 1.3-2.7, p=0.01).

The TM6SF2 homozygous wild-type allele was associated with increased cIMT (OR 3.4, 95% CI 1.3-5.8, p=0.004) in the lowest SMI tertile. In patients in the highest tertiles, the TM6SF2 variant was associated with EFT≥5.2 mm (OR 4.67, 95% CI 1.05-20.8, p=0.04). No significant differences in carotid plaques were found across genetic polymorphisms and SMI tertiles.

The TM6SF2 CT/TT variant was linked to a lower prevalence of dyslipidemia in the highest SMI tertiles (OR 0.20, 95% CI 0.08-0.50, p=0.001). Additionally, in the lowest SMI tertile, the TM6SF2 variant was associated with a lower prevalence of hypertension (OR 0.09, 95% CI 0.01-0.74, p=0.02) and lower WC (OR 0.25, 95% CI 0.08-0.84, p=0.02). The PNPLA3 GG variant is also associated with the female gender in both the lowest and highest SMI tertiles, with a strong effect in the lowest ones (lowest SMI OR 3.79, 95% CI 1.57-9.11, p=0.003; highest SMI OR 1.78, 95% CI 1.09-2.91, p=0.02). Moreover, the HSD17B13 wild-type variant is also associated with the female gender in the lowest SMI tertile (OR 3.23, 95% CI 1.20-8.66, p=0.02).

In the highest SMI tertile, polymorphisms in TM6SF2, MBOAT7, GCKR, and HSD17B13 were not associated with liver damage or laboratory parameters.

In Figure 2, we summarize the impact of genetic variants on CV and liver damage in patients with low muscle mass.

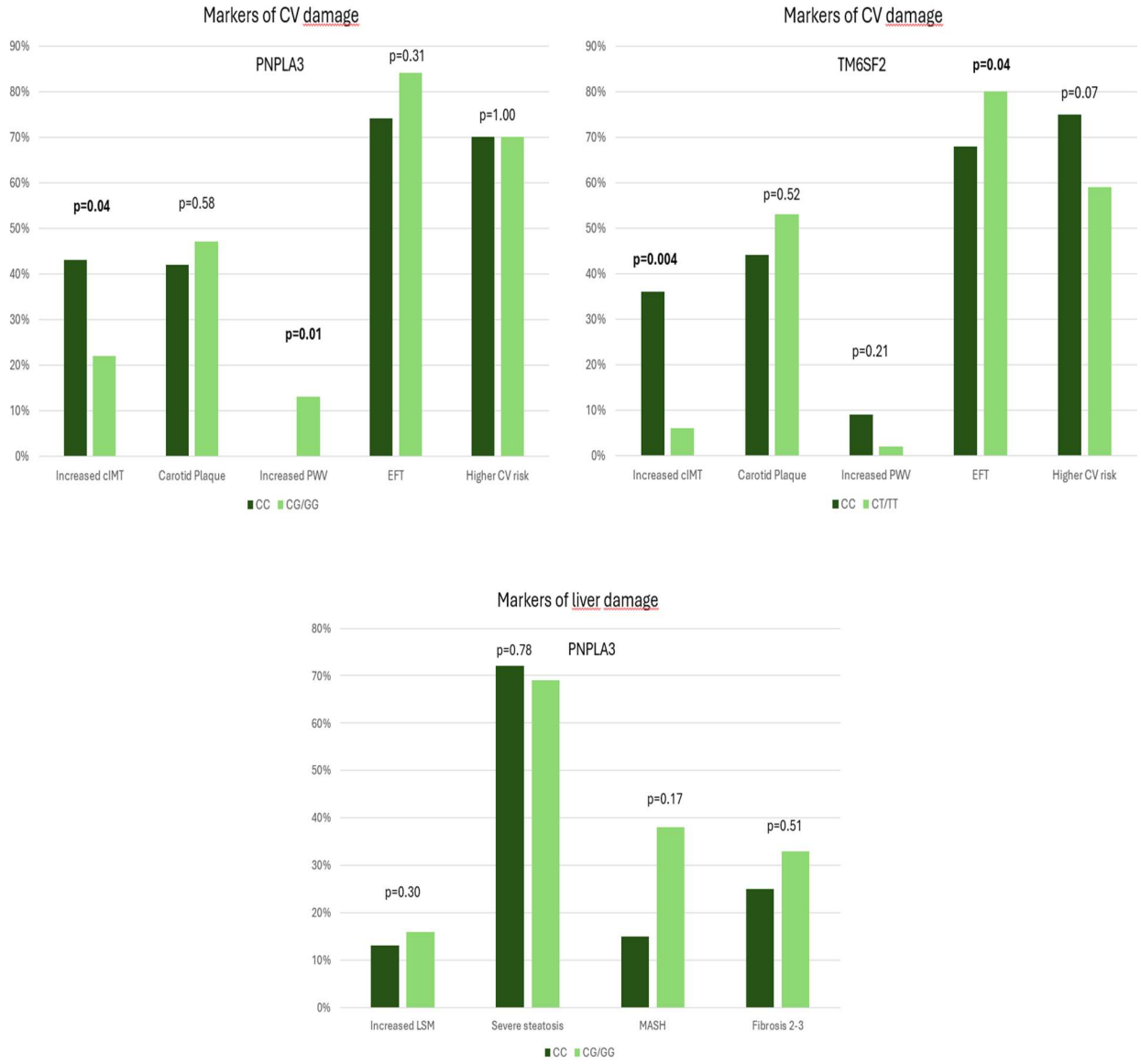

Figure 2. Association between genetic polymorphisms and marker of CV and liver damage in MASLD patients with low skeletal muscle mass.

3.5 Factors independently associated with low muscle mass in MASLD patients.

In the multivariate analysis, four distinct models were developed to assess the correlation between low SMI tertile with liver and CV damage.

In model 1, we examined the association between the lowest SMI tertile and age, gender, LSM, and increased cIMT. In this model, the lowest SMI tertile was significantly associated with LSM (OR 0.84, 95% CI 0.76-0.93, p=0.001), increased cIMT (OR 1.28, 95% CI 1.08-2.05, p=0.03), and age (OR 1.04, 95% CI 1.02-1.06, p=0.0002).

Model 2 focused on evaluating the relationship between liver damage, as expressed by liver fibrosis by LSM and low SMI tertile, adjusting for age, gender, BMI, WC, T2DM, increased ALT, and CAP. In this model, the lowest SMI tertile remained associated with low BMI (OR 0.62; 95% CI 0.55-0.70,

p<0.001), low WC (OR 0.91; 95% CI 0.87-0.95, p<0.001), with a modest association with age (OR 1.04; 95% CI 1.02-1.06, p=0.0003). Moreover, female was significantly associated with low SMI tertile (OR 2.32; 95% CI 1.40-3.85, p=0.001). Interestingly, in this model LSM remained associated with low SMI tertile (OR 0.91, 95% CI 0.87-0.95, p=0.0001) until adjustments were made for CAP and increased ALT.

Model 3 was designed to assess the correlation between CV damage as expressed by increased cIMT and low SMI tertile, with adjustment for age, gender, BMI, WC, T2DM, dyslipidemia, and smoke habits. In this model, the lowest SMI tertile was significantly associated with low BMI (OR 0.60; 95% CI 0.53-0.69, p<0.001), low WC (OR 0.92; 95% CI 0.87-0.96, p=0.0001), female (OR 2.19; 95% CI 1.32-3.64), and increased cIMT (OR 1.89; 95% CI 1.9-3.23).

Finally, Model 4 combined the parameters of both Model 1 and Model 2. In this unified model, the low SMI tertile remained significantly associated with low BMI (OR 0.61; 95% CI 0.53-0.71, p<0.001), low WC (OR 0.92; 95% CI 0.87-0.97, p=0.001), female (OR 2.21; 95% CI 1.23-4.00, p=0.008), and increased cIMT (OR 2.10; 95% CI 1.11-3.97, p=0.02), with a slight increase in age (OR 1.04; 95% CI 1.02-1.07, p=0.002).

The logistic regression models to evaluate factors associated with the lowest SMI tertile in MASLD patients were summarized in Table 3.

Table 3. Binomial logistic regression for low SMI tertile in MASLD patients.

	Model 1		Model 2		Model 3		Model 4	
Variable	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p-value	OR (95% CI)	p value
Age, years	1.04 (1.02-1.06)	**0.0002**	1.04 (1.02-1.06)	**0.0003**	1.03 (1.01-1.06)	**0.003**	1.04 (1.02-1.07)	**0.002**
BMI, kg/m2	-	-	0.62 (0.55-0.70)	**<0.001**	0.60 (0.53-0.69)	**<0.001**	0.61 (0.53-0.71)	**<0.001**
WC, cm	-	-	0.91 (0.87-0.95)	**<0.001**	0.92 (0.87-0.96)	**0.0001**	0.92 (0.87-0.97)	**0.001**
Female	1.04 (0.69-1.56)	0.87	2.32 (1.40-3.85)	**0.001**	2.19 (1.32-3.64)	**0.002**	2.21 (1.23-4.00)	**0.008**
T2DM	-	-	0.92 (0.52-1.62)	0.76	0.85 (0.42-1.70)	0.64	0.82 (0.37-1.80)	0.62
Increased ALT	-	-	0.80 (0.50-1.28)	0.35	-	-	0.78 (0.45-1.36)	0.38
CAP	-	-	0.99 (0.98-1.01)	0.84	-	-	1.01 (0.99-1.02)	0.94
LSM	0.84 (0.76-0.93)	**0.001**	0.96 (0.89-1.03)	0.24	-	-	0.94 (0.84-1.05)	0.30
Smoke habits	-	-	-	-	1.10 (0.60-2.01)	0.69	1.08 (0.54-2.18)	0.79
Increased cIMT	1.28 (1.08-2.05)	**0.03**	-	-	1.89 (1.09-3.23)	**0.02**	2.10 (1.11-3.97)	**0.02**
Dyslipidemia	-	-	-	-	1.29 (0.83-1.98)	0.25	1.10 (0.67-1.82)	0.70

Numbers in bold represent statistical significance.
In model 1, logistic regression was adjusted for age, gender, LSM, and increased cIMT. In model 2, logistic regression was adjusted for age, gender, BMI, WC, T2DM, elevated ALT, CAP, and LSM. In model 3, logistic regression was adjusted for age, gender, BMI, WC, T2DM, dyslipidemia, smoke habits, and increased cIMT. In model 4, the parameters of both Model 1 and Model 2 were combined.

Abbreviations: ALT: alanine aminotransferase, BMI: body mass index; CAP: controlled attenuation parameter; cIMT: carotid intima-media thickness; LSM: liver stiffness measurement; T2DM: type 2 diabetes mellitus; WC: waist circumference.

3.6 The impact of low muscle mass on liver and CV damage in subgroups of MASLD patients according to the presence of major metabolic alterations.

Given the high prevalence of metabolic comorbidities, mainly T2DM and hypertension, in patients with MASLD, we evaluated the association between SMI tertiles and markers of liver and CV damage and genetic predisposition in both hypertensive and diabetic patients.

3.6.1. Hypertensive patients.

Among the 841 MASLD patients, 352 (42%) had hypertension, of which 34% were in the lowest SMI tertile.

Hypertensive patients in the lowest SMI tertile, compared to others, exhibited significantly lower BMI (27.4 vs 30.9 vs 35.3 kg/m2, p<0.001), WC (101 vs 107 vs 114 cm, p<0.001), whereas were older compared to those in the highest tertile (59 vs 55 years, p=0.007).

These patients also demonstrated lower steatosis as measured by Fibroscan (mean CAP 300 vs 308 vs 324 dB/m, p=0.006), a reduced prevalence of advanced fibrosis (21% vs 34% vs 42%, p=0.003), and lower LSM values (5.2 vs 6.7 vs 6.7 kPa, p<0.001). Furthermore, patients in the lowest SMI tertile had a reduced prevalence of MASH compared to those in the highest tertile (48% vs 57%, p=0.03).

Regarding CV damage, hypertensive MASLD patients in the lowest SMI tertile showed a significantly higher prevalence of increased cIMT (34% vs 17% vs 25%, p=0.04) and PWV (24% vs 12% vs 9%, p=0.03), and of carotid plaques (52% vs 42%, p=0.04) compared to those in the higher SMI tertiles, but decreased EFT (7.5 vs 7.8 vs 8.4 mm, p=0.04).

Additionally, these patients had a lower prevalence of T2DM (34% vs 37% vs 51%, p=0.02), reduced rates of IR measured by HOMA-IR (57% vs 82% vs 85%, p=0.001), and lower rates of increased ALT (35% vs 53% vs 50%, p=0.02). Furthermore, a lower percentage of these patients carried the TM6SF2 CT/TT variant compared to those in the middle and higher tertiles (2% vs 20%, p=0.01). No other significant differences were observed across the SMI tertiles in this subgroup of patients.

The detailed characteristics of hypertensive MASLD patients by SMI tertiles are presented in Supplementary Table 1.

3.6.2. Diabetic patients.

In the MASLD cohort, 212 patients had also T2DM, of whom 29% in the lowest tertile. Diabetic patients in the lowest SMI tertile compared to patients in the highest SMI tertile were older (59 vs 54 years, p=0.01), had lower BMI (28.3 vs 35.8 kg/m2, p<0.001), and lower WC (103 vs 116 cm, p<0.001).

These patients also had lower CAP values (301 vs 329 dB/m, p=0.003), without any other differences in liver features.

Similarly, no difference in CV damage was seen across tertile, apart from a trend towards a higher prevalence of carotid plaque in the lowest tertile compared to the highest (61% vs 46%, p=0.20).

The detailed characteristics of diabetic MASLD patients by SMI tertiles can be found in Supplementary Table 2.

Given the small number of patients with both T2DM and low muscle mass, and the significant impact of T2DM on liver and CV damage, we focused our analysis on non-diabetic patients to better understand the effect of low muscle mass on liver and CV damage.

3.6.3. Non-diabetic patients.

Among non-diabetic MASLD patients, 36% were in the lowest SMI tertile, and these patients were older (52 vs 49 vs 45 years, p<0.001), had lower BMI (26.7 vs 29.2 vs 32.6 kg/m2, p<0.001), lower rates of obesity (11% vs 37% vs 69%, p<0.001), and reduced WC (99 vs 103 vs 108 cm, p<0.001) compared to those in the middle and highest tertiles. Moreover, non-diabetic patients in the lowest SMI tertile compared to those in the highest ones showed lower rates of IR (increased HOMA-IR 54% vs 80%, p<0.001), HDL (44 vs 51 mg/dL, p=0.0001), and lower increases of ALT levels (37% vs 59%, p<0.001), despite having elevating GGT (31% vs 23%, p=0.04), ferritin (43% vs 28%, p=0.03), LDL (129 vs 120 mg/dL, p=0.03) and dyslipidemia (54% vs 41%, p=0.02). In terms of liver damage, non-diabetic MASLD patients in the lowest SMI tertile exhibited less severe steatosis, with a lower prevalence of grade 3 steatosis on US (15% vs 24% vs 29%, p=0.02), and lower CAP values on Fibroscan (291 vs 300 vs 313 dB/m, p=0.002). As observed in the overall cohort and hypertensive patients, non-diabetic patients in the lowest SMI tertile also had lower LSM values (4.7 vs 5.3 vs 6 kPa, p<0.001), and a lower percentage of increased LSM (8% vs 16% vs 23%, p=0.0003). Notably, non-diabetic patients in the lowest SMI tertile demonstrated a higher prevalence of increased cIMT (26% vs 15% vs 19%, p=0.04) and carotid plaques (37% vs 28% vs 27%, p=0.04), alongside an elevated CV risk (58% vs 44% compared to those in the highest tertile, p=0.01). No differences in genetic polymorphisms were found.

Detailed characteristics of non-diabetic MASLD patients by SMI tertiles are presented in Supplementary Table 3.

3.6.4. Factors independently associated with low muscle mass in MASLD patients with hypertension, MASLD patients with T2DM, and non-diabetic MASLD patients.

We further investigated the relationship between low SMI and markers of CV and liver damage in hypertensive, in diabetic, as well as in MASLD patients without T2DM.

Low SMI in hypertensive MASLD patients was independently associated with increased cIMT (OR 2.06, 95% CI 1.07-3.93, p=0.03), cfPWV (OR 2.73, 95% CI 1.08-6.91, p=0.03), and a lower prevalence of increased ALT (OR 0.54, 95% CI 0.33-0.88, p=0.01). Additionally, low SMI was linked to the homozygous wild-type TM6SF2 variant in hypertensive patients.

In non-diabetic MASLD patients, low muscle mass was associated with increased cIMT (OR 1.66, 95% CI 1.04-2.65, p=0.03), carotid plaques (OR 1.56, 95% CI 1.07-2.27, p=0.02), and a reduced degree of fibrosis on histology (OR 0.29, 95% CI 0.08-0.98, p=0.04). Low SMI was also linked to lower ALT levels (OR 0.54, 95% CI 0.37-0.78, p=0.001), higher LDL levels (OR 4.38, 95% CI 1.28-15.0, p=0.02), and lower HDL levels (OR 1.03, 95% CI 1.01-1.05, p=0.002). Conversely, in diabetic MASLD patients, low SMI was not associated with markers of CV or liver damage, laboratory parameters, or genetic polymorphisms.

The logistic regression models assessing CV and liver damage, laboratory markers, and genetic factors associated with the lowest SMI tertile in hypertensive, diabetic, and non-diabetic MASLD patients are summarized in Table 4.

Table 4. Binomial logistic regression for low SMI tertile in hypertensive, diabetic and non-diabetic MASLD patients.

	Low SMI and hypertension		Low SMI and T2DM		Low SMI without T2DM	
	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
CV damage						
Increased cIMT	2.06 (1.07-3.93)	**0.03**	2.06 (0.69-6.17)	0.20	1.66 (1.04-2.65)	**0.03**
Increased cfPWV	2.73 (1.08-6.91)	**0.03**	1.57 (0.33-7.59)	0.57	1.49 (0.68-3.24)	0.32
Carotid plaques	1.25 (0.70-2.24)	0.44	1.63 (0.70-3.78)	0.25	1.56 (1.07-2.27)	**0.02**
EFT≥5.2 mm	0.74 (0.29-1.89)	0.53	3.08 (0.59-15.9)	0.18	1.27 (0.72-2.21)	0.41
High CV risk	1.25 (0.42-3.78)	0.47	1.06 (0.38-2.95)	0.22	1.39 (0.73-2.67)	0.32
Liver damage						
CAP	1.01 (0.99-1.02)	0.59	0.99 (0.98-1.01)	0.47	1.01 (0.99-1.02)	0.54
LSM	0.94 (0.86-1.02)	0.15	0.98 (0.92-1.05)	0.62	0.93 (0.85-1.01)	0.08
MASH	1.81 (0.26-12.5)	0.55	0.52 (0.16-1.73)	0.29	0.51 (0.16-1.65)	0.26
Fibrosis stage (histology)	0.79 (0.11-5.50)	0.81	0.55 (0.10-2.88)	0.11	0.29 (0.08-0.98)	**0.04**
Laboratory data						
Glycemia	0.99 (0.98-1.01)	0.36	0.99 (0.98-1.01)	0.35	1.01 (0.99-1.02)	0.12
Increased ferritin	0.81 (0.44-1.50)	0.51	0.59 (0.23-1.48)	0.26	1.47 (0.94-2.31)	0.09
Increased ALT	0.54 (0.33-0.88)	**0.01**	0.77 (0.41-1.46)	0.43	0.54 (0.37-0.78)	**0.001**
Triglycerides	0.99 (0.98-1.01)	0.33	0.99 (0.98-1.01)	0.40	0.99 (0.98-1.01)	0.75
LDL	1.01 (0.99-1.02)	0.18	1.62 (0.35-7.55)	0.54	4.38 (1.28-15.0)	**0.02**
HDL	1.01 (0.99-1.03)	0.24	1.02 (0.99-1.04)	0.18	1.03 (1.01-1.05)	**0.002**
Genetic polymorphisms						
PNPLA3 CG/GG	1.20 (0.58-2.48)	0.62	1.46 (0.43-4.94)	0.54	1.19 (0.74-1.92)	0.48
TM6SF2 CC	8.33 (1.07-64.9)	**0.04**	1.01 (0.31-3.28)	0.99	1.06 (0.56-1.98)	0.87
MBOAT7 CT/TT	0.84 (0.36-1.93)	0.68	1.91 (0.46-7.98)	0.38	1.11 (0.65-1.88)	0.70
GCKR CT/TT	1.96 (0.38-10.1)	0.42	2.95 (0.43-20.4)	0.99	1.10 (0.52-2.32)	0.80
HSD17B13 TTA/TATA	0.73 (0.32-1.69)	0.46	1.09 (0.31-3.82)	0.90	1.01 (0.60-1.71)	0.96

Numbers in bold represent statistical significance.
CV damage was adjusted for age, gender, smoke habits, and dyslipidemia.
Liver damage was adjusted for age, gender, BMI, and alcohol consumption.
Laboratory data and genetic polymorphisms were adjusted for age and gender.
Abbreviations: ALT: alanine aminotransferase; BMI: body mass index; CAP: controlled attenuation parameter; cfPWV: carotid-femoral pulse wave velocity; cIMT: carotid intima-media thickness; CV: cardiovascular; EFT: epicardial fat thickness; HDL: high density lipoprotein; LDL: low-density lipoprotein; LSM: liver stiffness measurement; MASH: metabolic-dysfunction associated steatohepatitis; WC: waist circumference.

3.7 Follow-up evaluation

In a subgroup of 104 patients with MASLD from the Milan cohort, we reassessed muscle mass, CV, and liver damage after a 5-year follow-up period.

3.7.1. Changes at follow-up independently of baseline SMI tertiles.

At follow-up, we observed a significant reduction in mean BMI (29.1 vs 28.4, p=0.001), with corresponding decreases in both overweight prevalence (49% vs 44%, p<0.001) and obesity rates (37% vs 34%, p<0.001), as well as a reduction in patients with increased WC (63% vs 58%, p<0.001). Muscle mass improved, as reflected by a decrease in the proportion of patients in the lowest SMI tertile (42% vs 22%, p<0.001), and an increase in those in the highest tertile (28% vs 42%, p<0.001). However, we noted an increase in all metabolic comorbidities, including T2DM, hypertension, and

dyslipidemia. Mean LDL levels were also lower (127 vs 105 mg/dL, p<0.001), likely due to a higher prescription rate of lipid-lowering drugs (10% vs 42%, p=0.001).

As for liver disease, fewer patients had elevated ALT (37% vs 30%, p=0.02) and GGT levels (28% vs 21%, p<0.001) at follow-up. Interestingly, 9% of patients showed a regression of steatosis at US, with a significant reduction in mean CAP (300 vs 289 dB/m, p=0.02). While mean LSM did not change, there was a decrease in the proportion of patients with LSM≥8 kPa (17% vs 7%, p<0.001).

Regarding CV damage, we observed a significant increase in carotid plaques (34% vs 54%, p<0.001), and mean EFT (7.5 vs 9.0 mm, p<0.001) and an increase in those categorized as having high CV risk (56% vs 63%, p=0.004).

Only 5% of patients experienced a worsening in SMI tertile from the middle to the low SMI tertile, while 74% remained in the same SMI tertile as at baseline, and 21% showed an improvement in their SMI tertile.

Table 5 summarizes the changes in anthropometric measures, laboratory results, liver and CV damage from baseline to follow-up.

Table 5. Changes in anthropometric measurements, laboratory data, markers of liver and CV damage from baseline to the 5-year follow-up.

	Baseline	Follow-up	p value
Anthropometric Features			
Alcohol consumption >Abstainers, N (%) >Low, N (%) >MetALD, N (%)	 68 (65) 31 (30) 5 (5)	 43 (41) 59 (57) 2 (2)	**0.04**
Active smokers, N (%)	23 (22)	17 (16)	0.23
Mean BMI, kg/m2 >Overweight, N (%) >Obese, N (%)	29.1±4.5 51 (49) 38 (37)	28.4±4.3 46 (44) 35 (34)	**0.001** **<0.001** **<0.001**
Mean WC, cm >WC>94/80 cm in M/W, N (%) >WC>102/88 cm in M/W, N (%)	102±10 92 (88) 66 (63)	102±10 92 (88) 60 (58)	0.49 1.00 **<0.001**
SMI tertiles >Low, N (%) >Middle, N (%) >High, N (%)	 44 (42) 31 (30) 29 (28)	 23 (22) 37 (36) 44 (42)	**<0.001**
Physical activity >Inactive, N (%) >Regular activity, N (%)	 63 (61) 41 (39)	 59 (57) 45 (43)	0.08
Metabolic Comorbidities			
T2DM, N (%)	16 (15)	23 (22)	**<0.001**
Hypertension, N (%)	48 (46)	54 (52)	**<0.001**
Dyslipidemia, N (%)	31 (30)	59 (57)	**<0.001**
Laboratory Data			
Fasting glucose, mg/dL	100±17	103±24	0.21
ALT, UI/L >Increased ALT, N (%)	33 [23-46] 38 (37)	27 [21-45] 31 (30)	0.87 **0.02**
AST, UI/L >Increased AST, N (%)	27 [21-31] 14 (13)	25 [21-32] 16 (15)	0.89 **0.036**
GGT, UI/L >Increased GGT, N (%)	30 [18-48] 29 (28)	24 [16-46] 22 (21)	0.12 **<0.001**
Ferritin, ng/mL	261 [151-474]	268 [127-510]	0.27

>Increased ferritin, N (%)	51 (49)	51 (49)	0.76
Triglycerides, mg/dL	136 [96-196]	131 [94-165]	0.06
LDL, mg/dL	127±28	105±37	**<0.001**
HDL, mg/dL	51±12	52±12	0.25
Liver Features			
US steatosis degree >0, N (%) >1, N (%) >2, N (%) >3, N (%)	 0 (0) 42 (41) 43 (41) 19 (18)	 9 (9) 36 (34) 51 (49) 8 (8)	**0.02**
CAP, dB/m	300±53	289±50	**0.02**
CAP degree >Mild, N (%) >Moderate, N (%) >Severe, N (%)	 8 (8) 8 (8) 73 (70)	 8 (8) 14 (13) 60 (58)	 0.51 0.59 **0.015**
LSM, kPa >LSM≥8kPa, N (%)	5.0 [4.1-6.4] 18 (17)	4.75 [4.1-5.7] 7 (7)	0.14 **<0.001**
CV Features			
cIMT≥0.9 mm, N (%)	22 (21)	43 (41)	0.08
Carotid plaques, N (%)	35 (34)	56 (54)	**<0.001**
Mean EFT, mm >EFT≥9.5/7.5 mm in M/W, N (%) >EFT≥5.2 mm, N(%)	7.5±2.0 29 (28) 94 (90)	9.0±2.3 55 (53) 101 (97)	**<0.001** 0.05 **0.03**
CV risk categories >Low, N (%) >High, N (%) >Very high, N (%)	 39 (38) 58 (56) 7 (6)	 38 (37) 66 (63) 0 (0)	**0.004**

Numbers in bold represent statistical significance.

Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; CAP: controlled attenuation parameter; cIMT: carotid intima-media thickness; CV: cardiovascular; EFT: epicardial fat thickness; GGT: gamma-glutamyl transferase; HDL: high density lipoprotein; LDL: low density lipoprotein; LSM: liver stiffness measurement; MetALD: metabolic alcohol-associated liver disease; SMI: skeletal muscle index; T2DM: type 2 diabetes mellitus; US: ultrasound; WC: waist circumference.

3.7.2. Changes at follow-up in patients with baseline low muscle mass.

We then compared the prevalence of metabolic alterations, liver, and CV damage at follow-up according to baseline SMI.

Patients in the lowest SMI tertile al baseline had a lower BMI at follow-up compared to those in the highest tertiles (26.3 vs 29.9 kg/m2, p<0.001), with a more modest reduction in mean BMI (-0.31 vs -1.04, p=0.05). Furthermore, patients in the low SMI tertile at baseline had a higher prevalence of dyslipidemia at the 5-year follow-up (73% vs 46%, p=0.01), although no differences were observed in the prevalence of other metabolic comorbidities.

In patients with low muscle mass at baseline, we observed a slight increase in mean LSM, whereas those with high muscle mass showed a reduction in mean LSM at follow-up (+0.47 vs -0.84 kPa, respectively, p=0.001).

No significant differences were observed in mean CAP and LSM≥8 kPa.

As for CV features, we did not notice any change over time in mean EFT, prevalence of carotid plaques, or increased cIMT according to baseline SMI.

3.7.3. Changes at follow-up in patients with persistent low muscle mass compared to those with improvement in muscle mass.

We then evaluated the prevalence of metabolic alterations, liver, and CV damage according to changes in SMI tertiles from baseline to follow-up.

Among patients with low muscle mass at both baseline and follow-up, compared to those with low muscle mass at baseline but an improvement at follow-up, we observed an increased prevalence of dyslipidemia (85% vs 70%, p=0.02) and carotid plaques (58% vs 40%, p=0.04) in those with persistent low muscle mass. However, no differences in other CV parameters or liver damage were found between patients with persistent low muscle mass and those with improved muscle mass.

3.7.4. Changes according to SMI tertile at follow-up.

Finally, we compared the prevalence of metabolic alterations, liver, and CV damage according to SMI at follow-up.

In patients with low skeletal muscle mass compared to those with increased muscle mass at follow-up, we observed a lower BMI (25.3 vs 29.6 kg/m2, p<0.001), a smaller proportion of obese patients (5% vs 43%, p=0.001), and lower WC (93 vs 104 cm, p<0.001), whereas a higher prevalence of dyslipidemia (85% vs 53%, p=0.01).

Regarding liver damage, patients with low muscle mass had higher prevalence of mild steatosis grades on abdominal US (steatosis grade 0-1: 69% vs 34%, p=0.007). Despite this, they experienced a slight increase in delta LSM over time, whereas those with higher muscle mass at follow-up showed a decrease in LSM over time (+0.98 [-0.6-1.15] vs -0.6 [-1.1-0.6], p=0.038). No significant differences were observed in markers of CV damage.

Supplementary Table 4 summarizes the differences in anthropometric data, metabolic comorbidities, laboratory results, and markers of liver and CV damage between patients in the low and high SMI tertile at 5-year follow-up.

Using Cox proportional hazard analysis, after adjusting for gender, age, physical activity, T2DM, and hypertension, baseline low muscle mass was significantly associated with changes in delta LSM (HR 2.30, 95% CI 1.38-3.81, p=0.013), BMI (HR 0.61, 95% CI 0.48-0.77, p=<0.001), and dyslipidemia (HR 5.74, 95% CI 1.62-20.3, p=0.007) at 5-year follow-up.

Figure 3 summarizes the hazard ratios (HR) and their corresponding 95% confidence interval (CI) illustrating the factors associated with baseline low muscle mass.

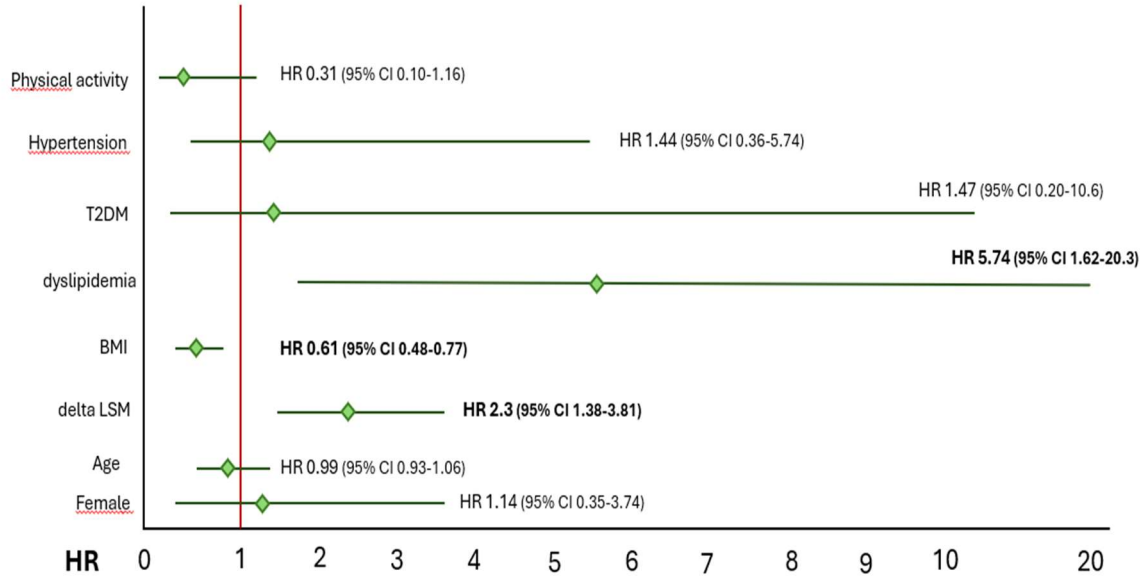

Figure 3. Hazard ratios (HR) and their corresponding 95% confidence interval (CI) of factors associated with baseline low muscle mass.
Abbreviations: BMI: body mass index; LSM: liver stiffness measurement; T2DM: type 2 diabetes mellitus.

4. DISCUSSION

Our study demonstrated a significant independent association between low muscle mass and increased risk of subclinical CV damage in non-cirrhotic MASLD patients. This finding is consistent with a previous study in 683 Korean NAFLD patients, which reported an increased prevalence of both cIMT and carotid plaques in NAFLD patients with low SMM assessed by BIA [109]. However, a key distinction between the Korean study and our research is the prevalence of obesity among patients enrolled. The Korean study observed a higher prevalence of obesity among patients with low SMM compared to those with higher SMM. In contrast, our study found that patients in the lowest SMI tertile had a lower percentage of obesity. One possible explanation is the lower cut-offs values used to define overweight and obesity in Asian populations compared to other ethnic groups, as previously mentioned [110].
The relationship between obesity and CV damage is well-established, particularly when associated with visceral adiposity, as indicated by increased WC [121]. Despite the low BMI and WC observed in our MASLD patients with low SMI, which suggests lower visceral adiposity, these patients still exhibited increased subclinical atherosclerotic damage. This finding reinforces the critical role of low muscle mass in CV damage, independent of obesity.
As previously noted, systemic inflammation and ROS linked both sarcopenia and CV disease [14]. Pro-inflammatory cytokines such as IL-6, TNFα, and C-reactive protein (CRP) contribute to reduced levels of IGF-1. In muscle, IGF-1 promotes protein synthesis through the phosphorylation of insulin receptor substrate 1 (IRS-1) and activation of phosphatidylinositide 3-kinases (PI3K)/protein kinase B (Akt) pathway. This pathway inhibits glycogen synthase kinase-3 (GSK3) and activates the mammalian target of rapamycin complex-1 (mTORC-1) [122], leading to increased muscle protein synthesis and decreased protein catabolism [123]. Conversely, reduced IGF-1 levels, together with oxidative stress, foster protein degradation through the ROS-induced NF-kB pathway [122]. This pathway increases myostatin levels, a muscle protein that inhibits muscle growth [124] by suppressing the AKT/mTOR axis and stimulating muscle proteasome and autophagy pathways, ultimately leading to sarcopenia [124].

Pro-inflammatory cytokines are also synthetized by cells within the atherosclerotic plaque, including macrophages, vascular smooth muscle cells, and endothelial cells [125]. These cytokines exacerbate endothelial dysfunction by promoting the proliferation of vascular smooth muscle cells and activating platelets [126]. This process leads to increased expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin, facilitating the transmigration of circulant monocytes into the subendothelium and contributing to plaque formation [125]. Additionally, impaired muscle perfusion [127] reduces the uptake of long-branched-chain amino acids and oxygen, further exacerbating muscle wasting [103] and increasing ROS production [128]. ROS-induced oxidation of LDL (oxLDL) not only promotes atherosclerotic plaque formation but also contributes, through the expression of endothelin-1 and the reduction of nitric oxide (NO) production, to endothelium constriction and hypertension [129].

It is important to note that the Korean study did not account for the degree of liver fibrosis in the patients enrolled and did not specify whether cirrhotic patients were excluded [109]. As fibrosis severity is an independent predictor of CV events in MASLD patients [130], with higher fibrosis correlating with increased subclinical atherosclerotic damage [131], our study focus on non-cirrhotic MASLD patients provides valuable insight. Our findings confirm that lower muscle mass is associated with increased subclinical atherosclerotic damage, independent of the degree of liver fibrosis, as evidenced by both Fibroscan and liver biopsy.

A large Korean study involving 7,191 patients from the KNHANES registry found that sarcopenia, assessed by DXA, was associated with increased CV risk in NAFLD patients, especially those with severe liver fibrosis [132]. However, this study had several limitations. First, both sarcopenia and liver fibrosis were evaluated using scores rather than imaging techniques. Additionally, the authors used the atherosclerotic cardiovascular disease (ASCVD) risk score and did not explore CV events or damage through imaging modalities.

Another relevant study conducted in China, which included 260 NAFLD patients aged over 45 years, found that while both lean and overweight NAFLD patients had a higher prevalence of carotid plaque, sarcopenia exerted an additional risk for carotid plaques only in lean NAFLD patients [133]. This supports the independent role of sarcopenia in CV damage. However, the majority of studies investigating the impact of sarcopenia on CV damage in MASLD patients have been conducted in Asian populations, which may limit the applicability of their findings to other populations. To date, our study is the first aimed to evaluate the impact of sarcopenia on CV damage in a European multicentric cohort of MASLD patients.

In our cohort, low muscle mass was associated with lower visceral adiposity, as indicated by lower BMI and WC. Previous studies have shown an association between sarcopenic obesity, a condition defined by the coexistence of obesity and sarcopenia, and MASLD, as well as the progression of liver fibrosis in these patients [134-136]. Obesity and sarcopenia share common pathogenetic mechanisms, including chronic inflammation, IR, oxidative stress, and alterations in pro-inflammatory cytokines and adipokines [137]. These mechanisms also contribute to MASLD and liver fibrosis, which may explain the observed association between sarcopenic obesity and MASLD [137]. Despite our findings seemingly contradicting the current literature, several factors may account for this discrepancy. First, most studies on sarcopenic obesity and MASLD were conducted in Asian population, where ethnicity affects body composition and BMI cut-offs for obesity differ from those used in Caucasian populations, nevertheless, a Chinese study indicated a higher prevalence of sarcopenia among lean NAFLD patients compared to others [133].

Additionally, a study conducted in Germany found that in patients with MASH, both fat mass and SMI were higher [138]. This suggests that increased fat mass, rather than sarcopenic obesity alone, may be associated with disease progression. A study conducted in Italy involving 680 elderly patients found that higher levels of body fat may be protective against the development of sarcopenia [139].

Moreover, some studies assessed steatosis using scores, that are less validated than abdominal US, the method used in our study. Although we observed a lower BMI in patients with low muscle mass, the mean BMI in the lowest tertile was 27 kg/m2, with 61% of patients classified as overweight. Given that MASLD diagnostic criteria include increased BMI and WC, a study conducted only on MASLD patients may introduce a bias in these measures.

We also observed a lower prevalence of T2DM in patients with the lowest SMI tertile compared to those with higher SMI. This finding appears contradictory given the association between sarcopenia and T2DM. Both conditions are age-related, and the prevalence of sarcopenia in diabetic patients ranges from 7% to 29% [8]. A meta-analysis of 6,526 patients found a higher prevalence of sarcopenia in T2DM patients compared to non-diabetic [140]. Factors such as age, lifestyle, endothelial dysfunction, low-grade systemic chronic inflammation, and oxidative stress are linked to both sarcopenia and T2DM [141]. Despite the strong association between T2DM and MASLD [142], only 25% of our cohort had T2DM. This may be due to the younger population in our study, which might underrepresent the impact of T2DM on muscle mass in MASLD. Notably, multivariate analysis showed no relationship between T2DM and muscle mass.

Given that MASLD is closely related to cardiometabolic risk factors, our study also points out the attention on hypertensive and diabetic MASLD patients. We found that hypertensive patients with low SMI had an increased risk of increased cIMT and PWV, both markers of CV damage.

Increased arterial stiffness is commonly observed among patients with obesity, particularly with high visceral adiposity [143]. The underlying mechanisms include dysregulation of adipokines production, systemic inflammation, ROS production, and IR [143], which are also implicated in sarcopenia. In our cohort, hypertensive MASLD patients showed a lower visceral adiposity compared to hypertensive patients with the highest SMI, further emphasizing the independent role of sarcopenia in arterial stiffness among MASLD patients with hypertension.

In our study, among diabetic MASLD patients we did not observe a significant relationship between low muscle mass and markers of liver and CV damage. Previous research has demonstrated that in patients with T2DM, the coexistence of sarcopenia and liver fibrosis is associated with increased arterial stiffness and endothelial dysfunction [144]. However, the role of sarcopenia alone in arterial stiffness remains controversial [145]. A prospective study of 852 diabetic patients followed for 6-8 years found that the presence of both sarcopenia and NAFLD increased the risk of carotid plaque progression, particularly in lean NAFLD patients [146]. However, this study did not re-evaluate the presence of sarcopenia and NAFLD at follow-up or account for metabolic changes during the study period.

In our cohort, we observed a trend towards a higher percentage of carotid plaque in diabetic patients with low muscle mass, though the small number of enrolled T2DM patients likely limited our ability to reach statistical significance. Interestingly, among non-diabetic MASLD patients, we found an independent association between low skeletal muscle mass and increased cIMT and carotid plaque, despite these patients having lower visceral adiposity and fibrosis compared to those with higher muscle mass. This suggests that sarcopenia plays an independent role in CV damage in non-diabetic patients, possibly because T2DM has a stronger influence on CV risk factors. Moreover, low muscle mass was associated with increased LDL levels in non-diabetic patients, reinforcing the association between muscle mass and lipid metabolism.

The accumulation of fat within muscle tissue, named myosteatosis, impairs mitochondrial function and reduces β-oxidation, thereby increasing ROS production [147]. This lipotoxicity promotes the production of pro-inflammatory cytokines, which exacerbate muscle protein breakdown and lead to muscle wasting [148]. Studies in elderly populations have similarly demonstrated higher levels of total cholesterol, LDL, TGs, and pro-atherogenic lipoproteins like VLDL and lipoprotein (a) in sarcopenic patients, highlighting the close relationship between sarcopenia and lipid disorders

[149,150]. Consistent with these findings, our study also observed a higher prevalence of dyslipidemia in patients with low muscle mass, corroborating a recent meta-analysis that demonstrated a direct correlation between sarcopenia and dyslipidemia (OR 1.47), including increased odds of elevated total cholesterol (OR 1.1), LDL (OR 1.95), and TGs (OR 30.13) [151].

To address the limitations of using SMI cut-offs not tailored for patients with CLD, we stratified our cohort by SMI tertiles based on gender. Janssen and colleagues in 2004 identified a SMI cut-off of <10.75 kg/m2 for men and <6.75 kg/m2 for women using BIA in a cohort of 4,499 elderly patients from the United States [21]. In our study, we found a similar cut-off for men (SMI <10.35 kg/m2), but the cut-off for women was significantly higher than that proposed by Janssen (7.75 vs 6.75 kg/m2), which may explain the association between low muscle mass and female gender in our cohort.

The gender differences in sarcopenia remain a subject of debate. While some studies report a higher prevalence of sarcopenia in men [152,153], others highlight a stronger association between sarcopenia and metabolic syndrome in women [154]. These differences may be attributed to different muscle phenotype in men and women, as men tend to have more glycolytic fibers, whereas women have a greater proportion of oxidative fibers, which have higher mitochondria content [155]. Glycolytic fibers are more vulnerable to muscle wasting, while oxidative fibers are more affected by disuse muscle atrophy [156]. A recent study conducted on murine model showed that mitochondrial dysfunction, oxidative stress, and autophagy mediated by the AMPK pathway are more pronounced in sarcopenic male mice compared to females [157], suggesting different pathogenetic mechanisms of sarcopenia in each gender.

Testosterone, through its interaction with androgen receptors, promotes muscle protein synthesis [158], and its age-related decline contributes to IR, increased visceral adiposity, reduced IGF-1, and ultimately, protein catabolism and muscle wasting [159]. In contrast, estrogen supports muscle regeneration by promoting the differentiation of satellite cells into muscle fibers and exerts antioxidant effects on muscle tissue [160]. The decline in estrogen during menopause contributes to muscle mass loss in women, albeit at a slower rate compared to the muscle decline seen in men [161]. The majority of females in our cohort were post-menopausal, which likely accounts for the observed association between female gender and low muscle mass.

Interestingly, we observed a lower degree of liver damage among patients with low muscle mass. This finding contrasts with existing literature, which indicates that sarcopenia is closely linked to liver fibrosis, steatohepatitis, and steatosis in biopsy-proven NAFLD patients, independent of other metabolic risk factors [64,162]. The association between sarcopenia and liver damage is likely due to shared pathogenic mechanisms, including IR, chronic low-grade systemic inflammation, increased myostatin levels, and dysregulation of IGF-1 signaling and mitochondrial function [132]. The impact of sarcopenia on liver fibrosis appears to be additive in the presence of NAFLD, particularly for significant fibrosis rather than cirrhosis [62]. However, most studies examining the relationship between sarcopenia and fibrosis in patients with steatosis are cross-sectional, which limits the ability to establish a cause-effect relationship. Additionally, the role of muscle fat infiltration (myosteatosis) in liver disease progression has gained increasing attention. Myosteatosis, rather than muscle mass loss, has been shown to correlate more strongly with liver fibrosis [66,163] and steatohepatitis [70], particularly in patients with obesity. Prospective studies have also confirmed the association between myosteatosis and both liver inflammation and fibrosis [66,164,165].

In our study, we excluded patients with biopsy-confirmed cirrhosis or clinical/US suspicion of cirrhosis. The majority of our patients (76%) had a LSM of less than 8 kPa, and 52% of those who underwent liver biopsy had no or mild fibrosis. Furthermore, the lower BMI and WC of patients with low muscle mass could explain the reduced severity of steatosis, steatohepatitis, and fibrosis observed in these patients. Multivariate analysis also did not reveal an independent relationship

between liver damage markers and low skeletal muscle mass after the adjustment for major confounders, likely due to the lower visceral adiposity, milder steatosis, and reduced prevalence of T2DM among patients with low SMI in our cohort.

Finally, we explored the role of genetic polymorphisms, known to be associated with liver damage in MASLD patients, with sarcopenia and CV damage. To our knowledge, this is the first study to specifically evaluate the impact of genetic variants on CV damage in MASLD patients, according to the presence of sarcopenia.

In our cohort of MASLD patients, we did not observe any association between genetic variants in PNPLA3, TM6SF2, MBOAT7, GCKR, and HSD17B13 genes and muscle mass. A 2019 study involving 3,969 Chinese patients found an inverse correlation between muscle mass and the presence of PNPLA3 wild-type and heterozygous variants, but not in patients with the mutated GG variant, who were less susceptible to developing NALFD despite low muscle mass [166]. One possible explanation is that carriers of the PNPLA3 GG variant express higher levels of irisin, as demonstrated in a study of 481 children [167]. Irisin is known to improve both muscle and liver insulin sensitivity, potentially reducing the progression of liver disease and muscle wasting [53]. However, another study of 401 Chinese males found that the combination of low muscle mass and the PNPLA3 GG variant increased the risk of steatosis and fibrosis in biopsy-proven NAFLD patients [168]. Another study assessing the impact of PNPLA3 and TM6SF2 variants on liver fibrosis in NAFLD patients concluded that only the PNPLA3 variant heightened susceptibility to liver fibrosis, with a risk greater than that conferred by low muscle mass [169].

Beyond liver damage, the role of PNPLA3 in CV damage in MASLD patients remains inconclusive. A Japanese study showed that the PNPLA3 wild-type CC variant was linked to an increased risk of CV disease [170], while a study based on the NHANES registry found that individuals with the PNPLA3 GG genotype had higher overall and CV-related mortality in both the general population and NAFLD patients [171]. In contrast, a study among MASLD patients with T2DM reported that neither PNPLA3 nor TM6SF2 polymorphisms were associated with the development of CV events [172].

In our study, we identified an association between the PNPLA3 genetic variant and increased cfPWV across the entire cohort, as well as in patients with low muscle mass. Interestingly, the PNPLA3 CG/GG variant appeared to be linked to a lower cIMT in patients with low SMI. Conversely, the TM6SF2 CT/TT variant was associated with lower CV damage markers, particularly reduced cIMT and a lower prevalence of hypertension, both in the overall cohort and in patients with low muscle mass. Furthermore, the TM6SF2 variant was associated with a lower incidence of dyslipidemia, lower BMI, and reduced TGs levels.

Several previous studies have demonstrated the protective effect of the TM6SF2 variant on CV damage [173], which was further confirmed by a Mendelian randomization study [174]. Carriers of TM6SF2 variant exhibited a more favorable lipid profile, with reduced total cholesterol, LDL, and TGs levels [175], along with elevated HDL levels [176]. In patients with MASH, TM6SF2 carriers also showed a favorable lipid profile, with a lower prevalence of carotid plaques and CV events compared to wild-type patients, as reported by Dongiovanni and colleagues [177]. Additionally, these patients showed lower CRP, indicating lower inflammation levels [178].

Among other genetic variants analyzed, both GCKR and HSD17B13 polymorphisms were associated with increased CV risk in the entire cohort of MASLD patients, with the GCKR variant also associated with a higher prevalence of hypertension. Previous research has connected the GCKR variant with CAD, mediated through elevated levels of pro-atherogenic lipoprotein such as sdLDL, triacylglycerols, and reduces HDL levels [179]. However, the role of HSD17B13 loss-of-function variant in CV damage remains unclear.

Moreover, we found a higher prevalence of the PNPLA3 GG variant and the HSD17B13 loss-of-function variant in female patients with low skeletal muscle mass. A recent multicenter study

showed an additive effect between female sex and the PNPLA3 variant on the risk of steatosis, fibrosis, and HCC development in patients at risk of SLD [180]. Estrogens appears to upregulate PNPLA3 expression, as observed in women during the follicular phase of the menstrual cycle, where higher estrogen levels were associated with increased PNPLA3 mRNA levels [180]. This heightened prevalence of the PNPLA3 variant among female patients in our cohort may help explain the association between female sex and low SMI, although further research is needed.

Finally, we observed a significant association between the PNPLA3 genetic variant and increased LSM in patients within the highest SMI tertile, potentially accounting for the elevated LSM values seen in these patients in our study.

When we reassessed a subgroup of MASLD patients from the baseline cohort, we observed a reduction in mean BMI, leading to a decrease in overweight and obese patients. Additionally, 9% of these patients experienced a resolution of liver steatosis. Although we did not observe a statistically significant difference in physical activity, likely due to the limited number of patients reevaluated, a trend toward increased physical activity from baseline to follow-up was noted. Notably, all patients had been provided with a nutritional plan at baseline, which was reviewed at each follow-up visit. The observed reduction in mean BMI over time can likely be attributed to these lifestyle changes, implemented and monitored through regular follow-up in our Metabolic Clinic.

We also noted improvements in muscle mass among reassessed patients and a reduction in mean CAP values. However, despite these positive trends, we observed an increase in the prevalence of metabolic comorbidities such as hypertension, T2DM, and dyslipidemia. Consequently, we observed an increase in CV damage markers, particularly carotid plaques and EFT, and an increase in CV risk.

Interestingly, despite a reduction in visceral adiposity, especially in patients with low muscle mass at follow-up, there was an increase in the percentage of patients with advanced fibrosis as measured by Fibroscan. When stratifying patients by baseline muscle mass, we found that patients with low muscle mass at baseline experienced a worsening in mean LSM, while those with higher muscle mass at baseline showed improvements in mean LSM over time. Moreover, persistent low muscle mass at follow-up was associated with worsening liver damage, further emphasizing the link between low muscle mass and fibrosis progression.

As stated before, previous studies investigating the relationship between sarcopenia and liver fibrosis were cross-sectional. Most cohort studies have focused on the impact of sarcopenia on mortality or liver-related events in cirrhotic patients [181-183]. Among studies aimed to evaluate the impact of sarcopenia on liver damage in earlier stages of CLD, a study conducted on 521 biopsy-proven NAFLD patients found a higher prevalence of significant fibrosis (stage 2-4) in patients with low muscle mass, particularly when accompanied by myosteatosis and increased visceral adiposity [184]. Conversely, a longitudinal study conducted in Korea suggested that liver fibrosis may accelerate muscle mass loss over time [42].

Our findings confirm the relationship between low muscle mass, lower BMI, and a higher prevalence of dyslipidemia. Patients with baseline low muscle mass had a lower BMI at follow-up and a higher prevalence of dyslipidemia. Moreover, in patients who maintained low muscle mass at both baseline and follow-up, compared to those who showed improvements in muscle mass, we observed an increase in carotid plaques, indicating increased CV damage.

We also confirmed a higher prevalence of females among patients with low muscle mass at follow-up, which was associated with lower steatosis degree and a lower BMI.

Despite the increased prevalence of metabolic comorbidities at follow-up, particularly T2DM, which could contribute to worsening liver stiffness, baseline low muscle mass remained significantly associated with higher LSM, as well as lower BMI and a greater incidence of dyslipidemia.

Although dyslipidemia was more prevalent in patients with low muscle mass, we did not observe an independent association between baseline low muscle mass and markers of CV damage at follow-

up. This lack of association is possibly due to the relatively short follow-up period, as a longer duration may be necessary to detect more pronounced CV damage.

This study has several strengths. First, its multicenter design, involving patients of European ancestry, enhances the generalizability of the findings within this population. Liver damage was rigorously assessed using both abdominal US and Fibroscan for all patients, with confirmation through liver biopsy in a subset of patients, further validating the results among liver damage. Moreover, more than half of the cohort underwent direct CV damage assessment through carotid US, and a significant portion also had evaluations of arterial stiffness and EFT. Importantly, the majority of patients enrolled had a comprehensive genetic profiling, including variants known to be associated with liver damage. The longitudinal nature of the study, with assessments of body composition, liver, and CV damage at both baseline and follow-up, strengthens the ability to infer a causal relationship between sarcopenia and liver and CV damage.

Nevertheless, the study has several limitations. The evaluation of muscle mass in non-cirrhotic patients with MASLD using BIA is not yet standardized, and specific SMI cut-offs for patients with CLD remain undefined. However, the EWGSOP2 guidelines support the use of BIA in large clinical studies due to the absence of radiation and the low cost [18]. Another limitation is the absence of data on sex hormone levels, particularly among female patients, as well as missing information on muscle strength and performance, both of which are key components for diagnosing sarcopenia. At baseline, all patients were provided with a nutritional plan, however, in this study we did not assess their food intake using a validated questionnaire. Finally, although this was a longitudinal study, the follow-up analysis was conducted on only a small subset of the cohort, which may limit the statistical power of the cause-effect relationship identified.

Although sarcopenia is associated with increased mortality, regardless of the population studied or the method used to assess it [4], current recommendations for preventing sarcopenia focus primarily on a diet rich in branched-chain amino acids and a combination of aerobic and resistance exercises [185]. To date, vitamin D supplementation and the use of anabolic hormones are not recommended for the treatment of sarcopenia [185].

5. CONCLUSION

In conclusion, our multicentric study identified an independent relationship between low muscle mass, assessed through a non-invasive, radiation-free method, and increased markers of atherosclerotic damage in non-cirrhotic MASLD patients, particularly in those who were hypertensive and non-diabetic. Interestingly, we observed a lower prevalence of liver damage in patients with low muscle mass, possibly due to their lower visceral adiposity.

However, in the longitudinal phase of the study, we found an independent association between low muscle mass and increased mean LSM, indicating that low muscle mass predisposes patients to liver damage over time. Persistent low muscle mass also appeared to increase CV damage, although we did not observe an independent association, likely due to the shorter follow-up period.

While most studies report a higher prevalence of sarcopenia in males, our findings highlighted a significant link between low muscle mass and females, potentially related to hormonal changes affecting muscle mass and body composition after menopause.

Genetic predisposition also plays a key role in the impact of sarcopenia on liver and CV damage in MASLD patients. In our cohort, the TM6SF2 polymorphism was associated with lower CV damage, suggesting a protective role against CV disease. Conversely, genetic variants in GCKR and HSD17B13 were linked to higher CV damage. Notably, the PNPLA3 variant and the HSD17B13 loss-of-function variant were more common in females, while the PNPLA3 variant was associated with increased LSM in patients with high muscle mass, partially explaining the higher LSM values observed in this group.

By incorporating imaging techniques to assess both liver and CV damage, along with genetic profiling, this study offers a more comprehensive understanding of the relationship between sarcopenia and CV damage in non-cirrhotic MASLD patients. Maintaining muscle mass is crucial for preventing both liver and CV diseases, and future public health campaigns should prioritize promoting muscle mass health. However, further studies with longer follow-up are required to confirm the relationship between sarcopenia and CV damage in MASLD patients. Future research should also evaluate the long-term impact of genetic variants and identify those that may predispose MASLD patients to sarcopenia. Finally, dietary patterns and hormonal changes should be considered to gain a deeper understanding of the complex relationship between muscle mass and both liver and CV health.

6. ABBREVIATIONS

ALD: alcohol-associated liver disease.
AKT: protein kinase B.
ALT: alanine aminotransaminase.
AMPK: AMP-activating protein kinase.
ASCVD: atherosclerotic cardiovascular disease.
AST: aspartate aminotransaminase.
BIA: bioelectrical impedance analysis.
BMI: body mass index.
CAC: coronary artery calcium.
CAD: coronary artery disease.
CAP: controlled attenuation parameter.
cfPWV: carotid-femoral pulse wave velocity.
cIMT: carotid intima-media thickness.
CLD: chronic liver disease.
CRP: C-reactive protein.
CT: computed tomography.
CV: cardiovascular.
DXA: dual-energy X-ray absorptiometry.
EASL: European Association for the Study of Liver disease.
EFT: epicardial fat thickness.
ELF: enhanced liver fibrosis.
ESC: European Society of Cardiology.
EWGSOP: European Working Group on Sarcopenia in Older People.
FFAs: free fatty acids.
FFM: free fat mass.
GCKR: glucokinase regulator.
GGT: gamma-glutamiltransferase.
GLUT4: glucose transporter type 4.
GSK3: glycogen synthase kinase-3.
GWAS: genome-wide association studies.
HCC: hepatocellular carcinoma.
HDL: high-density lipoprotein.
HOMA-IR: homeostatic model assessment for insulin resistance.
HSD17B13: 17β-hydroxysteroid dehydrogenase type 13.
HSI: hepatic steatosis index.
ICAM-1: intercellular cell adhesion molecule-1.

ICD-10: International Classification of Disease.
IGF-1: insulin-like growth factor-1.
IL: interleukin.
IQR: interquartile range.
IR: insulin resistance.
IRS-1: insulin receptor substrate 1.
LDL: low-density lipoprotein.
LSM: liver stiffness measurement.
MASH: metabolic dysfunction-associated steatohepatitis.
MASLD: metabolic dysfunction-associated steatotic liver disease.
MBOAT7: membrane bound O-acyltransferase domain containing 7.
MetALD: metabolic alcohol-associated liver disease.
MRI: magnetic resonance imaging.
mTORC-1: mammalian target of rapamycin complex-1.
NAFLD: non-alcoholic fatty liver disease.
NAS: NAFLD activity score.
NFkB: nuclear factor-kB.
NO: nitric oxide.
oxLDL: oxidized low-density lipoprotein.
PI3K: phosphatidylinositide 3-kinases.
PNPLA3: patatin-like phospholipase domain-containing 3.
PWV: pulse wave velocity.
ROS: reactive oxygen species.
SD: standard deviation.
sdLDL: small-dense low-density lipoprotein.
SLD: steatotic liver disease.
SMI: skeletal muscle index.
SMM: skeletal muscle mass.
SNP: single nucleotide polymorphism.
SREBP-1c: sterol regulatory element-binding protein-1c.
TBW: total body water.
T2DM: type 2 diabetes mellitus.
TGs: triglycerides.
TM6SF2: transmembrane 6 superfamily member 2.
TNFα: tumor necrosis factor-α.
US: ultrasound.
VCAM-1: vascular cell adhesion molecule-1.
VCTE: vibration-controlled transient elastography.
VLDL: very low-density lipoprotein.
WC: waist circumference.

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