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Non-alcoholic fatty liver disease: liver, muscle, and gut interactions

JOANA DE SÁ ALVES RIGOR

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Advisor:

Professora Doutora Daniela Maria Martins Mendes, MD, PhD

External researcher – Faculdade de Medicina da Universidade do Porto

Associate Professor – Faculdade de Ciências da Saúde da Universidade Fernando Pessoa

Coadvisor:

Professor Doutor Pedro Barata Coelho, MD, PhD

Associate Professor – Faculdade de Ciências da Saúde da Universidade Fernando Pessoa

Exma. Senhora

Mestre Joana de Sá Alves Rigor

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TEL +351 22 040 8000. FAX +351 22 040 8186/ 8187
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Abbreviations and acronyms

1H-MRS – single-voxel proton magnetic resonance spectroscopy

25[OH]D – 25-hydroxyvitamin D

AdipoR1 – Adiponectin receptor 1

AGR – android-fat-to-ginoid-fat ratio

AHR – adjusted hazard ratio

ALT – alanine aminotransferase

AMPK – AMP-activated protein kinase

AOR – adjusted odds ratio

APRI – aspartate aminotransferase to platelet ratio index

ASM – appendicular skeletal muscle mass

AST – aspartate aminotransferase

BARD – body mass index, aspartate aminotransferase/alanine aminotransferase ratio, and diabetes score

BIA – bioelectrical impedance analysis

BMI – body mass index

CAP – controlled attenuation parameter

CD – cluster of differentiation

CHB – chronic hepatitis B

CHD – coronary heart disease

CLD – chronic liver disease

CNS – comprehensive NAFLD score

COPD – chronic obstructive pulmonary disease

CRP - C-reactive protein

CT - computed tomography

CVD – cerebrovascular disease

DXA – dual-energy x-ray absorptiometry

EFS – elbow flexor strength

eGFR – estimated glomerular filtration rate

EWGSOP – European Working Group on Sarcopenia in Older People

F – fibrosis grade

FABP – fatty acid binding proteins

FATP – fatty acid transport proteins

FFM – fat-free mass

FGF21 – fibroblast growth factor 21

FIB-4 – Fibrosis-4 index

FLI – Fatty Liver Index

FM – fat mass

FNDC5 – fibronectin type-III domain containing protein 5

FOXO1 – forkhead box protein O1

FPG – fasting plasma glucose

FT4 – free thyroxine

G3 – grade 3

GDCA – glycodeoxycholic acid

GGT – gamma-glutamyltransferase

GLUT4 – glucose transporter type 4

GUDCA – glycoursodeoxycholic acid

HbA1c – glycated hemoglobin

HDL-c – high-density cholesterol

HFS – hepamet fibrosis score

HGS – handgrip strength

HIV – human immunodeficiency viruses

HOMA-IR – homeostasis model of insulin resistance

hsCRP – high sensitivity C-reactive protein

HSI – Hepatic Steatosis Index

HT – hypertension

IL– interleukine

INSTI – integrase strand transfer inhibitor

IQR – interquartile range

IR – insulin resistance

IRS-1 – insulin receptor substrate-1

ISI – insulin sensitivity index

KES – knee extension strength

KNHANES – Korean National Health and Nutrition Examination Survey

L3 – third lumbar vertebrae

L4 – fourth lumbar vertebrae

LB – liver biopsy

LDL-c – low-density lipoprotein cholesterol

LFS – liver fat score

LILACS – Latin American and Caribbean Health Sciences Literature

LPS – lipopolysaccharides

LSM – liver stiffness measurement

MAFLD – metabolic associated fatty liver disease

MetS – metabolic syndrome

MMA – midthigh muscle area

MRI – magnetic resonance imaging

mRNA – messenger ribonucleic acid

mTOR – mammalian target of rapamycin

NA – not applicable

NAFL – nonalcoholic fatty liver

NAFLD – nonalcoholic fatty liver disease

NAS – NAFLD activity score

NASH – non-alcoholic steatohepatitis

NF-κB – nuclear factor kappa B

NFLS – NAFLD Liver Fat Score

NFS – nonalcoholic fatty liver disease fibrosis score

NHANES – National Health and Nutrition Examination Survey

NNRTI – non-nucleoside reverse transcriptase inhibitors

NR – not reported

NRTI – nucleoside reverse transcriptase inhibitors

NS – non significant

OR – odds ratio

PGC-1α – peroxisome ɣ coactivator-1α

PI – protease inhibitor

PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analysis

PROSPERO – International Prospective Register of Systematic Reviews

Q1 – lowest quartile

Q4 – highest quartile

SBP – systolic blood pressure

SCFA – short-chain fatty acids

SD – standard deviation

SI – sarcopenia index

SITT – short insulin tolerance test

SMA – skeletal muscle area

SMI – skeletal muscle index

SMM – skeletal muscle mass

SPPB – Short Physical Performance Battery

T1 – lowest tercile

T2DM – type 2 diabetes mellitus

T3 – highest tercile

TβMCA – tauro-β-muricholic acid

Th – T helper cells

TLR – Toll-like receptors

TUDCA – tauroursodeoxycholic acid

Thesis outline

This Thesis has been structured as follows:

In the "**Abstract**", a summary of the Thesis is presented.

In **Chapter 1, "Rationale"**, the motivations behind the Thesis are explained, as well as their relevance for explaining aspects of the pathophysiology of non-alcoholic fatty liver disease (NAFLD) and helping in disease staging.

In **Chapter 2, "Aims"**, the main objectives of the Thesis are described.

In **Chapter 3,** "**Background**", the theoretical concepts and previous research that contextualizes the Thesis are presented. In **Chapter 3.1, "The Metabolic Syndrome and the Liver"** the links between the Metabolic Syndrome (MetS) and NAFLD and the gaps still existing in NAFLD pathophysiology are explained. In **Chapter 3.2, "Non-alcoholic Fatty Liver Disease: prevalence, diagnosis, and staging"** the epidemiology of NAFLD and current issues regarding its diagnosis and staging are defined. In **Chapter 3.3, "Muscle health, sarcopenia, and its effects on metabolic health"** the evidence of the association of muscle health with metabolic health are reviewed, known links between the two are described, and the recommended methods for the assessment of muscle mass, muscle strength, and physical performance are explained. In **Chapter 3.4, "Myokines as keys to muscle function and signaling"**, the concept of myokines and the actions of the most significant ones are summarized. In **Chapter 3.5, "Microbiome in the crosstalk between gut, liver, and skeletal muscle"** the composition and influence of the gut microbiome on overall health, metabolic health, NAFLD, and sarcopenia are explained.

In **Chapters 4** to **8** the results of the research produced are described.

In **Chapter 4, "Noninvasive fibrosis tools in NAFLD: validation of APRI, BARD, FIB-4, NAFLD fibrosis score, and Hepamet fibrosis score in a Portuguese population"**, a multicenter retrospective cohort study of liver biopsy patients is presented, in which the noninvasive tools mentioned in the title are validated for the exclusion of advanced liver fibrosis in Portuguese patients.

In **Chapter 5, "How sarcopenia, muscle mass, muscle strength, and physical performance relate to non-alcoholic fatty liver disease: a systematic review"**, a systematic review analyses the associations of NAFLD and NAFLD severity with muscle mass, muscle strength, physical performance, and sarcopenia.

In Chapter 6, "Associations between muscle mass, strength, and performance and non-alcoholic fatty liver disease", a cohort study of patients with MetS cared for in a tertiary hospital center outpatient clinic is described, with focus on the variables associated with NAFLD and NAFLD severity, particularly ones concerning the muscle parameters previously stated.

In **Chapter 7, "Fibroblast growth factor 21 and myostatin are higher in females with NAFLD and correlate with dysmetabolism and lower muscle mass, strength, and performance"**, in a cohort study, the association of NAFLD and serum myokines and adipokines is presented, and these cytokines are correlated to biochemical and body composition parameters.

In **Chapter 8, "Gut microbiome composition and its associations with NAFLD and low muscle mass"**, the gut microbiome composition of a cohort population is reported, and associations with NAFLD and with low muscle mass are analyzed.

In **Chapter 9, "Conclusions and future research"**, the main conclusions, limitations and strength of each study are summarized, and thoughts on avenues for future research are laid out.

List of publications

Core research papers

By order of appearance in the thesis:

1. **Rigor J**, Diegues A, Presa J, Barata P, Martins-Mendes D
   Noninvasive fibrosis tools in NAFLD: validation of APRI, BARD, FIB-4, NAFLD fibrosis score, and Hepamet fibrosis score in a Portuguese population
   Postgrad Med 2022;134(4):435-440

2. **Rigor J**, Monteiro-Soares M, Barata P, Martins-Mendes D
   How sarcopenia, muscle mass, strength, and performance relate to non-alcoholic fatty liver disease: a systematic review
   [under review at Obesity Research & Clinical Practice]

3. **Rigor J**, Vasconcelos R, Lopes R, Moreira T, Barata P, Martins-Mendes D
   Associations between muscle mass, strength, and performance and non-alcoholic fatty liver disease
   Minerva Gastroenterol (Torino) 2022;10.23736/S2724-5985.22.03097-2

4. **Rigor J**, Luís C, Rocha AC, Fernandes R, Barata P, Martins-Mendes D
   FGF21 and myostatin are higher in females with NAFLD and correlate, respectively, with dysmetabolism and lower muscle mass, strength, and performance
   [submitted to Journal of Hepatology]

5. **Rigor J**, Rocha AC, Barata P, Martins-Mendes D
   Bifidobacterium abundance is reduced in patients with low muscle mass that do not have NAFLD
   [submitted to Porto Biomedical Journal]

Other work

Oral presentations:

1. "*Prémio propina de doutoramento 2019 - apresentação de projeto*", presented at "Reunião Papel da Medicina Interna no Ensino Médico e Investigação", Lisbon, January 25th, 2020
2. "*Apresentação das bolsas vencedoras 2019: Bolsa Helena Saldanha / Boehringer Ingelheim e Bolsa NEDM/ Lilly*", presented at "GLUCO STORM – Highlights da Diabetes 2020", virtual event, November 28th 2020

In the Non-alcoholic Fatty Liver Disease topic but not included in this thesis:

3. **Rigor J**, Grupo de Hepatologia do Serviço de Medicina Interna do CHVNG/E, Martins-Mendes D
   "Ratio neutrófilos/linfócitos e a sua associação com fibrose na doença de fígado gordo não alcoólico"
   XIII Jornadas do Núcleo de Estudos das Doenças do Fígado, Porto, October 4-5th 2019
   [poster presentation]

4. **Rigor J**, Luís C, Barata P, Martin-Mendes D
   "Ratio de gordura andróide-ginóide associado com a doença de fígado gordo não alcoólico independentemente de outros parâmetros metabólicos"
   24º Congresso Português da Obesidade, virtual event, November 2020
   [poster presentation]

5. Martins B, **Rigor J**, Rocha AC, Dias MC, Luis C, Baylina P, Martins-Mendes D, Fernandes R
   "Stress oxidativo em diabéticos com fígado gordo não-alcoólico"
   Revista Portuguesa de Diabetes. 2021; 16 (1) Suppl: 83
   [poster abstract]

In the Sarcopenia topic but not included in this thesis:

6. **Rigor J**, Barbosa JP, Luís C, Fernandes R, Barata P, Martins-Mendes D
Low skeletal muscle function, but not mass, is associated with the presence of type 2 Diabetes
Revista Portuguesa de Diabetes. 2019; 14 (4): 173
[poster abstract]

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The following awards were received:

- **Best poster presentation** for "*Ratio neutrófilos/linfócitos e a sua associação com fibrose na doença de fígado gordo não alcoólico"* at the "*XIII Jornadas do Núcleo de Estudos das Doenças do Fígado"*, Porto, October 4-5th 2019
- **Doctorate Tuition Award** (*Prémio Propina de Doutoramento*) **2019**, awarded by the Portuguese Society of Internal Medicine (*Sociedade Portuguesa de Medicina Interna*)

Abstract

Background

NAFLD is the most common liver disease worldwide. It is associated with MetS, type 2 diabetes mellitus (T2DM), and obesity. However, much of its pathophysiology needs clarification, particularly the mechanisms behind differing prevalence and natural history in populations with the same risk factors. One of the most important steps in the treatment and follow-up of patients with NAFLD is the accurate staging of NAFLD fibrosis, which may be done using appropriately validated non-invasive clinical tools.

Cytokines are signaling proteins that aid communication between cells, neighboring or at a distance. Some of the most studied cytokines are those produced by adipose tissue, also known as adipokines. Like the adipose tissue, the skeletal muscle also produces specific cytokines, called myokines. Both adipokines and myokines communicate with other organs, namely the liver, gut, heart, and brain. As such, both the adipose and the skeletal muscle are endocrine organs.

Exercise has been shown to promote metabolic health past weight loss. In contrast, sarcopenia, or low muscle strength accompanied by low muscle mass, with or without low physical performance, has been associated with different illnesses, such as cancer and autoimmune disorders. Some evidence has suggested that sarcopenia is more prevalent in patients with NAFLD and more prevalent with increasing NAFLD severity.

The gut microbiome is the biggest population of symbiotic microbes in the human body. It has important functions in digestion, metabolism, and immunity. Dysbiosis, or dysfunction of the microbiome, has been implicated in a plethora of diseases, including obesity, T2DM, and NAFLD, although evidence is still conflicting in the latter. Few studies have investigated the association of muscle parameters and gut microbiome.

Aims

The main purposes of this research were to better characterize the association of NAFLD and NAFLD severity with muscle strength, muscle mass, and physical performance, and to investigate possible links between liver, muscle, and gut via the expression of myokines and the gut microbiome.

Material and Methods

The following studies were performed:

1) A validation study, for the Portuguese population, of the aspartate aminotransferase (AST) to Platelet Ratio Index (APRI); the Fibrosis-4 Index (FIB-4); the NAFLD fibrosis score (NFS); the body mass index (BMI), aspartate aminotransferase/alanine aminotransferase ratio, and diabetes score (BARD); and the Hepamet Fibrosis Scoring System (HFS) – **Chapter 4**.
2) A systematic review on the associations between NAFLD and NAFLD severity, and sarcopenia, muscle mass, muscle strength, and physical performance – **Chapter 5**.
3) A cohort study of patients with MetS followed in a tertiary hospital center, characterizing body composition, muscle and biochemical parameters, and liver steatosis, and determining variables associated with NAFLD and NAFLD severity – **Chapter 6**.
4) A cohort study of the association of specific myokines and adipokines with NAFLD and their correlation to muscle and other biochemical parameters – **Chapter 7**.
5) A sub-analysis of a sample the cohort population characterizing the gut microbiome according to NAFLD and muscle mass – **Chapter 8**.

Results

APRI, BARD, FIB-4, NFS, and HFS are adequate tools for the exclusion of advanced fibrosis in a Portuguese population, with a negative predictive value (NPV) 89.9%-96.4% and area under the receiver operating characteristic curve (AUROC) 0.80-0.88, allowing for their use both in the clinical setting as well as in research studies.

We found evidence in the literature that low muscle mass and low muscle strength were associated with NAFLD and NAFLD severity. However, the quality of the evidence was limited by the variety of tools used, particularly in the assessment of muscle mass, that precluded a meta-analysis.

In our population of patients with MetS, we found an association between NAFLD and low appendicular skeletal mass (ASM), as measured by dual-energy x-ray absorptiometry (DXA) and indexed to weight. Analysis of serum myokines showed that, in females, higher concentrations of fibroblast growth factor 21 (FGF21) and myostatin were found in patients with NAFLD. FGF21 correlated with insulin resistance, high triglycerides (TG), low high-density lipoprotein cholesterol (HDL-c), high BMI, high fat percentage, low muscle mass, and low physical performance, while myostatin correlated with low muscle mass, low muscle strength, and low muscle performance. Regarding the gut microbiome, patients overall had less relative quantity of most bacterial populations analyzed, except for *Proteobacteria*. In patients without NAFLD, those with low muscle mass had a lower relative abundance of *Bifidobacterium*, and, when not considering this group, patients with NAFLD had a trend towards a lower relative abundance of this genus.

Conclusions

Evidence from present literature and from our cohort studies indicates that an association between the presence of NAFLD and low muscle mass, and possibly strength and physical performance, exists. In females, the myokines FGF21 and myostatin may be a link between these variables as they associate with the presence of NAFLD and correlate with different muscle parameters. Differences were also perceived in gut microbiome populations, particularly the reduced abundance of *Bifidobacterium* in patients without NAFLD in those that presented low muscle mass, and, in those with normal muscle mass, a tendency to lower abundance of this same genus for those with NAFLD, which may indicate a role in both NAFLD and muscle health.

1. Rationale

NAFLD is a rising health concern, particularly in developed countries and in people with obesity, insulin resistance, T2DM, and dyslipidemia.(1) Even though it affects over 25% of the population, there are still many questions to be answered regarding its pathophysiology, diagnosis, and management.(1, 2)

The natural history of NAFLD is extremely variable from one individual to another.(3) While some patients shown no progress of the disease for decades, others progress to cirrhosis and hepatocellular carcinoma, with poor prognosis and the need for liver transplant.(3) Advanced fibrosis is the most important predictor of developing significant liver disease.(4) Traditionally, fibrosis staging required a liver biopsy; however, the implementation of vibration-controlled transient elastography (VCTE) and of non-invasive staging tools allows for the exclusion of patients unlikely to have advance fibrosis.(4) While non-invasive tools are far more accessible than VCTE, they have not, however, been validated for the Portuguese population.

The pathophysiology of NAFLD is thought to be multifactorial in nature; the most supported theory is one that states that multiple insults, acting together or in sequence, lead to its development and progression.(5) Although several of these insults, like insulin resistance and the activation of the immune system, have been described, they have not yet been able to fully explain the different prevalence and natural history of NAFLD in patients that present the same risk factors.(2, 5)

Muscle health and the positive effects of exercise in the promotion and restoration of health as a whole, and of metabolic health in particular, have gained growing interest.(6) The loss of muscle strength and muscle mass, with or without low physical performance, commonly known as sarcopenia, is a process associated with age, but also with disease, particularly of the inflammatory type.(7) Much like the adipose tissue and its adipokines, the skeletal muscle functions as an endocrine organ that produces specific cytokines, known as myokines for their origin, that have been shown to produce changes in the bone, liver, pancreas, heart, and brain.(8) Regarding NAFLD, recent evidence has proposed that sarcopenia is more common in patients with NAFLD, and more common with increased severity of NAFLD.(9, 10) These findings suggest a link between this disease and muscle metabolism, where myokines may serve as communication.

The gut microbiome may be another factor bridging the interaction between all these organs. The gut microbiome is the largest symbiotic population of bacteria, that inhabits our body.(11) It has functions in digestion, but also in metabolism and immunity, and its dysbiosis has been found to affect several organs, like the liver and brain.(12) Recent research has found specific patterns of microbiome populations in patients with obesity, T2DM and NAFLD; yet, evidence is still, at times, conflicting.(13, 14) In the field of muscle health and microbiome, very few studies in humans have been reported.(15)

2. Aims

The main purposes of this Thesis are to better characterize the association of NAFLD and NAFLD severity with muscle strength, muscle mass, and physical performance, and to investigate possible links between liver, muscle, and gut via the expression of myokines and the gut microbiome.

Specific objectives for each study included:

1) To validate non-invasive tools for exclusion of advanced fibrosis in a Portuguese population, allowing their subsequent use in this work and in clinical practice in general – **Chapter 4**.
2) To summarize and critically analyze previous evidence on the associations between NAFLD, NAFLD severity, and muscle parameters – **Chapter 5**
3) To characterize the association of muscle mass, muscle strength, and physical performance, with NAFLD and NAFLD severity – **Chapter 6**.
4) To describe the pattern of serum myokines and adipokines in patients with NAFLD and to correlate this pattern with metabolic abnormalities and with muscle mass, muscle strength, and physical performance – **Chapter 7**.
5) To analyze associations of gut microbiome relative populations with muscle parameters and NAFLD – **Chapter 8**.

3. Background

3.1. The Metabolic Syndrome and the Liver

The MetS is a cluster of disorders related to overfeeding, that parallels economic development worldwide, with an estimated prevalence of a quarter to a third of the western population.(16) Several definitions for MetS have been proposed, all including a combination of several features, namely abdominal obesity, T2DM or insulin resistance, hypertension (HT), low HDL-c, and high TG.(17) While some controversy exists regarding the pertinence of classifying as a syndrome these individual conditions,(18) there are some common underlying pathophysiological mechanisms, and clustering of cardiovascular risk factors is an established phenomenon that contributes to overall cardiovascular disease and disability.(17)

MetS is a multisystemic illness and NAFLD can be regarded as its hepatic manifestation.(19) NAFLD is defined as the intrahepatic accumulation of lipids without other known etiology, and should be suspected in patients with MetS, obesity, or T2DM.(3) The close relationship of NAFLD and dysmetabolism has motivated a suggested change of nomenclature to Metabolic Associated Fatty Liver Disease (MAFLD).(20, 21) In this new definition, instead of relying on the exclusion of other causes of liver disease, the diagnosis is established by the presence of hepatic steatosis and either obesity, T2DM, or at least two metabolic risk abnormalities.(20)

Although NAFLD is known to be directly connected to excess weight and insulin resistance, its exact pathophysiology is still not completely understood.(2) The most popular theory is the “multiple hit” theory, in which several insults, working simultaneous or sequentially, lead to fatty acid accumulation and peroxidation, recruitment and activation of inflammatory cells, fibrosis with subsequent architectural distortion, and activation of oncogenesis.(5, 22)

3.2. Non-alcoholic Fatty Liver Disease: prevalence, diagnosis, and staging

As the prevalence of MetS grows in a population, so does the prevalence of NAFLD; as such, in Europe and North America, estimates point to around 25% of the population being affected.(1) The spectrum of NAFLD is wide, from simple steatosis to steatohepatitis and even cirrhosis.(23) While simple steatosis is a benign and highly prevalent condition, inflammation and fibrosis carries a significant risk of morbidity and mortality, both from cardiovascular disease and from liver related complications.(3) Therefore, and in the absence of specific therapies, the cornerstone of management is the adequate identification of patients with or at risk of advanced NAFLD.(23)

Liver biopsy is the gold-standard for NAFLD diagnosis and staging. However, given that most patients with NAFLD do not progress to advanced stages of fibrosis and cirrhosis, in clinical practice, biopsy is generally foregone in exchange for clinical diagnosis and non-invasive fibrosis staging tools.(4). VCTE uses pulse-echo ultrasound to measure the velocity of a sheer mechanical wave across the liver, yielding a liver stiffness measurement estimate that correlates well with histological fibrosis.(24) VCTE use depends on the availability of the equipment, which still has significant costs, and requires operator training; these factors limit its widespread use.

Several scores and tests that use easily available clinical and laboratory data have been created. (4) These scores have been designed to exclude patients that are unlikely to have advanced fibrosis and that, as such, will most likely not benefit from extensive testing and close follow-up.(4) The ease of use of these scores allows for their application in primary care and in lower resource settings. The use of multiple tools, either systematically or sequentially, can provide more accurate results, as has been proposed by several authors.(4, 25) These tools APRI,(26, 27) FIB-4,(28, 29) NAFLD fibrosis score,(30) BARD,(31) and Hepamet Fibrosis Scoring System,(32) while universally accessible, have not been validated for all populations, including the Portuguese one.

3.3. Muscle health, sarcopenia, and its effects on metabolic health

It has been well established that physical exercise is key to overall health. This is particularly true in patients with MetS, where exercise is able to reverse every component of this syndrome.(6) Interestingly, the positive effect of exercise on an individual's glycemic and lipid profile goes beyond what would be expected to result from the exercise-induced weight loss.(6) This points to an independent effect of exercise and muscle health on metabolic health as a whole.

Disorders of the skeletal muscle can be characterized in various ways. Sarcopenia has previously been defined as loss of muscle mass and function/strength,(33) while in most recent guidelines, strength has assumed the forefront as the most reliable parameter of muscle health.(7) Despite these classifications, the term "sarcopenia" has been extensively used in research as a surrogate of low muscle mass alone.(33) In clinical practice, sarcopenia is not necessarily immediately apparent, particularly when it is shrouded by obesity, a condition called "sarcopenic obesity".(34)

Sarcopenia has traditionally been regarded as a disease of the elderly. Aging is characterized by several cellular alterations, namely immunosenescence, mitochondrial dysfunction, accumulation of damaged protein, epigenetic changes and telomere shortening, that lead to multisystemic changes.(35) There are many parallels between the process of aging and dysmetabolism. For example, both aging and obesity are considered chronic inflammatory processes, that activate macrophages, T cell lymphocytes, and mast cells, and lead to the release of cytokines.(36)

In the muscle, insulin appears to be a potent anabolic promoter. It stimulates protein synthesis by increasing messenger ribonucleic acid (mRNA) translation and prevents protein lysis by stabilizing lysosomes and reducing the activity of the ubiquitin-proteasome pathway.(37) Insulin resistance impairs this mechanism and contributes to sarcopenia.

Both obesity and T2DM affect mitochondrial function in the skeletal muscle, mostly by oxidative stress and lipid peroxidation.(38, 39) Ectopic deposition of fat occurs with insulin resistance, as insulin suppresses fatty acid oxidation in the muscle

tissue.(39) In patients with T2DM, a reduction in type 1 muscle fibers, which are mitochondria rich and have a high oxidative capacity, and an increase in type 2 fibers, which are glycolytic and less insulin sensitive, have been shown.(39)

Testosterone and growth hormone are anabolic and contribute to protein synthesis, increased insulin-like growth factor 1 and decreased inflammatory cytokines.(37) Obesity is associated with lower levels these hormones, contributing to sarcopenia.(37) Neuro- and vasculopathy, and the accumulation of advanced glycation end-products, secondary to T2DM, also contribute to muscle tissue loss and dysfunction.(40)

The relationship between sarcopenia and MetS is bidirectional. The muscle is the largest organ involved in glucose metabolism; as such, loss of muscle mass directly impacts overall insulin resistance.(38) Myosteatosis activates serine/threonine kinases that antagonize insulin signaling, contributing to insulin resistance.(39, 41) In a systemic way, loss of muscle mass and strength leads to exercise intolerance, which perpetuates the cycle of sarcopenia and MetS.(39)

For the purpose of diagnosis and research, several instruments have been developed and validated for the assessment of muscle mass, strength, and physical performance.(7) To quantify muscle mass, bioelectrical impedance analysis (BIA) and imaging tools, like computed tomography (CT), magnetic resonance imaging (MRI) and dual-energy x-ray absorptiometry (DXA) can be used.(7, 42) BIA, where locally available, is a popular and cost-efficient method.(43) However, it does not measure muscle mass directly, but yields an estimate via whole-body electric conductivity, making its use not recommended in research studies.(7) Techniques using CT or MRI scans analyze either whole-body muscle mass or a segment of muscle area. They are limited by availability, cost, and in the case of CT, radiation exposure.(42) DXA allows for the differentiation between different tissues by calculating their attenuation of radiation, dividing the body in three compartments: bone, lean mass, and fat mass.(44) Appendicular skeletal muscle mass (ASM) can be derived from lean mass measurements from the limbs as, in this compartment, lean tissue is essentially muscle.(45) DXA is the most commonly used device for body composition analysis as it uses a small dose of radiation, is relatively low cost, and takes less than 20 minutes for a whole-body scan. (42, 45)

Muscle strength can be assessed in the upper or lower body by measurement of the grip strength or by a chair stand test, respectively.(7) Both methods correlate with overall strength; however grip strength requires a validated dynamometer,(46) while a chair stand test only necessitates a stop watch to time how long it takes a patient to stand up and sit back down 5 times.(47) Balance and gait speed tests were added to the chair stand test, creating a composite test called the Short Physical Performance Battery (SPPB), which serves to assess physical performance.(47) The SPPB can be employed in most settings with very little material (a chair, a stopwatch, and a measuring tape), and is recommended for use in both clinical practice and research studies.(7, 47)

3.4. Myokines as keys to muscle function and signaling

Myokines are peptides produced in the muscle that exert autocrine, paracrine, and endocrine functions.(48) Their receptors exist in multiple organs, including adipose tissue, liver, heart, bone, and brain, varying for each specific myokine.(8) Myokines are thought to be responsible for the beneficial multisystemic effect of exercise.(48) There are about 3000 myokines, including interleukine-6 (IL-6), fibronectin type-III domain containing protein 5 (FNDC5)/irisin, myostatin, myonectin, fibroblast growth factor 21 (FGF21), and adiponectin. (8, 48)

IL-6 is a well research cytokine, expressed by numerous types of cells, including macrophages, fibroblasts, adipocytes and myocytes.(48) IL-6 has a somewhat paradoxical behavior: while it is a pro-inflammatory cytokine, it is also mediates the beneficial metabolic effects of both acute and chronic exercise by promoting the expression of IL-10 and IL-1 receptor antagonist and blocking the production of tumor necrosis factor α (TNF-α) and IL-1bβ, thus creating an anti-inflammatory milieu.(49, 50) While IL-6 levels in adipose cells are correlated with dysmetabolism and inflammation markers,(36) IL-6 from skeletal muscle stimulates lipolysis and lipid oxidation and has been show to mediate the exercise induced reduction in visceral fat mass.(50) IL-6 regulates the proliferation of muscle satellite cells leading to skeletal muscle hypertrophy.(36) However, in patients with cardiovascular disease, particularly in men, IL-6 has been associated with lower muscle mass and strength.(51, 52) NAFLD patients have been found to have higher baseline IL-6 levels (53), even when comparing to other obese individuals;(54) higher levels have also been found non-alcoholic steatohepatitis (NASH) when comparing with simple steatosis.(55)

FNDC5 is a transmembrane protein that is expressed in the skeletal muscle tissue, the heart and the brain.(56) In the past 10 years, this protein has gained interest for its association with muscle health and metabolism.(56, 57) The ectodomain of FNDC5, irisin, is cleaved in response to exercise and then travels to adipose tissue, where it increases the expression of several brown fat associated genes that intervene in thermogenesis, namely peroxisome ɣ and its coactivator-1α (PGC-1α) and mitochondrial uncoupling protein mRNA 1 (UCP1).(36, 57) In the liver, FNDC5/irisin acts upon activated

hepatic stellate cells, attenuating their migration and reducing their contractibility and proliferation, while also ameliorating inflammatory cytokine expression.(58) It also inhibits hepatic lipogenesis and maintains the balance in hepatic glucose metabolism.(8) In humans, irisin levels are lower in sarcopenia (59, 60) as well as in NAFLD (61, 62), with an inverse relationship with intrahepatic TG content(63); however, these findings have not been consistent across the literature.(61, 64)

Myostatin belongs to the transforming growth factor-β (TGF-β) superfamily of proteins and plays a negative role in regulating muscle mass. (59) It modulates Akt pathway activity, inhibiting protein synthesis through the mammalian target of rapamycin (mTOR) and increasing muscle atrophy via the forkhead box protein O1 (FOXO1) pathways.(36) It has a role in insulin resistance by nuclear factor kappa B (NF-κB) and smad3 activation(65) and is also involved in the browning of white adipose tissue (WAT).(8, 36) However, tendentially, lower myostatin serum levels have been associated with low muscle mass and function,(66-68) with possible sex differences.(69, 70) A single human study has found higher levels of myostatin in women with NAFLD compared to those without.(71)

Myonectin, one of the most recently described myokines, has been associated with lipid and glucose metabolism.(8, 36) In muscle, it suppresses the transcription of autophagy genes and activates Akt, insulin receptor substrate-1 (IRS-1), and mTOR.(48) In the liver, myonectin enhances fatty acid uptake by hepatocytes by inducing the expression of cluster of differentiation (CD36), fatty acid transport proteins (FATP), and fatty acid binding proteins (FABP) in these cells.(8) Myonectin has an inverse relationship with BMI,(72, 73) T2DM,(74) insulin resistance,(72-75) levels of TG,(72, 73) and of cholesterol.(73) A study showed an increase in myonectin levels in females subjected to a 8-week aerobic exercise program.(76)

FGF21 is secreted in many organs, namely fat, liver and muscle, acting as an adipokine, hepatokine, and myokine.(36) In an animal study, under conditions of stress, FGF21 was involved in the removal of damaged mitochondria in muscle through mitophagy.(77) In adipose tissue, similarly to irisin, FGF21 regulates thermogenesis, by PGC-1α and UCP1 expression promotion.(36) It also plays a part in insulin metabolism, in liver, muscle and pancreas, by improving insulin sensitivity and restoring β-cell

function.(78) In humans, in the presence of insulin resistance, T2DM and MetS, muscle FGF21 levels are elevated.(8) High levels of FGF21 have also been associated with NAFLD and correlated with hepatic TG content. (79, 80)

As both an adipokine and a myokine, adiponectin regulates the interaction of muscle, fat, and pancreas.(8) It promotes myogenesis, inhibits proteolysis and regulates skeletal muscle through AMP-activated protein kinase (AMPK)-stimulated glucose transporter type 4 (GLUT4) translocation and lipid oxidation.(81) Adiponectin receptor 1 (AdipoR1) is the main receptor present in skeletal muscle and is reduced in obese patients, contributing to sarcopenia.(59) In most studies,(82-85) though not in all,(86) circulating adiponectin is higher in sarcopenic compared to non-sarcopenic individuals. In NAFLD, meta-analyses have shown it to be decreased in simple steatosis, and even more so in the presence of hepatic fibrosis.(87) Adiponectin is frequently studied alongside leptin, another adipokine, as the ratio of these two molecules has a stronger correlation in MetS and T2DM.(88) Persistent increase in leptin levels is proposed to decrease responsiveness of pancreatic β-cell receptors, leading to increased insulin secretion and resistance.(88) In direct opposition to adiponectin, leptin levels are increased in patients with NAFLD and with increased severity of disease,(89) and are decreased in patients with sarcopenia.(83, 84)

3.5. Microbiome in the crosstalk between gut, liver, and skeletal muscle

The human microbiome is composed by all the microbes, be them bacteria, viruses, protozoa, or fungi, that coexist in and with our organism.(11, 12) It is a dynamic and complex population that has evolved alongside the human species for thousands of years.(11) Of the several microbiomes present in the human body, the gut microbiome is the largest, with more than 1500 species distributed in more than 50 different phyla, the most common being *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*.(12) While an individual's gut microbiome is generally stable from the age of 2 years-old, the balance of proportion between different phyla is sensitive to diet composition and its changes, particularly alterations in fat and fiber content. (90) Dysfunctions of the microbiome, also known as dysbiosis, have been associated with a multitude of diseases, including neoplastic, autoimmune, and metabolic.(91)

The gut microbiome has important roles in metabolism and immunity, with effects that extend beyond the gastrointestinal tract.(14) Bacteria in the gut are responsible for the digestion of bile acids, fermentable carbohydrates, and protein, among others.(92) Certain bile acid derivatives, such as tauroursodeoxycholic acid (TUDCA), tauro-β-muricholic acid (TβMCA), glycoursodeoxycholic acid (GUDCA), and glycodeoxycholic acid (GDCA) have been shown to improve glucose metabolism by inducing insulin sensitivity in the liver and muscle and by reducing hyperglycemia and glucose intolerance. (92) The metabolism of poorly absorbed carbohydrates produces short-chain fatty acids (SCFA), which lessen hepatic fat infiltration and insulin resistance.(92) In the absence of these carbohydrates, the microbiome in the distal colon resorts to protein fermentation, leading to metabolites such as ammonia and branched-chain fatty acids. These metabolites induce inflammation of the mucosa and subsequently increase gut permeability, allowing for the systemic passage of endotoxins.(92)

Mucosal immunity is in large part assisted by the interactions between the gut microbiome and the host.(93) Lipopolysaccharides (LPS) on the surface of gram-negative bacteria induce a immune response by activating Toll-like receptors (TLR), which

mediate LPS translocation into intestinal capillaries. (13, 90) Higher LPS has been found to be associated with higher blood TG and lower HDL-c, and with insulin resistance.(90) The microbiome modulates cellular immunity via the maturation of T cells, namely T helper 17 (Th17) and CD8+ cells.(94) It may also influence the diversity of antigens derived from immunoglobulin A (IgA) + B cells in the gut.(94) SCFA indirectly lowers production of proinflammatory cytokines such as TNF-α, IL-6, and IL-12, while some bile acid metabolites induce production of IL-22. (92)

Studies in animals and humans have produced evidence of different pattern of microbiome population distribution in health and in various disease states. Overall, a decrease gut microbial diversity has been associated with obesity and T2DM.(95) In obesity, higher abundance of *Firmicutes* and lower abundance of *Bacteriodetes* has been described, as well as reduced *Bifidobacterium*.(96) In individuals with NAFLD, relative increases have been found in *Escherichia*, *Prevotella* and *Streptococcus* and decreases in *Coprococcus*, *Faecalibacterium,* and *Ruminococcus*. (97, 98) However, across studies, many discrepant findings still exist, possibly related to distinct populations characteristics (such as ethnicity and dietary habits), to NAFLD diagnosis and staging differences, and to microbiome sequencing methods. (14)

In the field of sarcopenia, there is still scarce evidence of microbiome differences. Lower muscle strength, as grip strength, has been associated with higher relative abundance of *Proteobacteria*, *Sutterella*, *Clostridium,* and *Holdemania,* and lower relative abundance of *Faecalibacterium*, while lower physical performance, as gait speed, has been associated with higher relative abundance of *Enterobacteriaceae*.(15) Studies in rats have pointed to an effect of *Faecalibacterium* in increasing muscle mass, (15) and this finding has been replicated in an observational study of children.(99)

4. Noninvasive fibrosis tools in NAFLD: validation of APRI, BARD, FIB-4, NAFLD fibrosis score, and Hepamet fibrosis score in a Portuguese population

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Noninvasive fibrosis tools in NAFLD: validation of APRI, BARD, FIB-4, NAFLD fibrosis score, and Hepamet fibrosis score in a Portuguese population

Joana Rigor [a,b], Andreia Diegues [c], José Presa [d], Pedro Barata [e,f,g] and Daniela Martins-Mendes [b,g,h,i]

[a]Internal Medicine Department, Vila Nova de Gaia/Espinho Hospital Centre, Vila Nova de Gaia, Portugal; [b]Biomedicine Department, Faculty of Medicine of the University of Porto, Porto, Portugal; [c]Internal Medicine Department, Unidade Local de Saúde do Nordeste, Bragança, Portugal; [d]Liver Unit, Internal Medicine Department, Trás-os-Montes e Alto Douro Hospital Centre, Vila Real, Portugal; [e]Faculdade de Ciências da Saúde da Universidade Fernando Pessoa, Porto, Portugal; [f]Pathology Department, Centro Hospitalar Universitário do Porto, Porto, Portugal; [g]Inovação em Saúde, University of PortoI3S – Instituto de Investigação e , Porto, Portugal; [h]Internal Medicine Department, Hospital Escola da Universidade Fernando Pessoa, Porto, Portugal; [i]LaBMI - Biotech solutions, PORTIC - Porto Research, Technology & Innovation Center, Polytechnic Institute of Porto, Porto, Portugal

ABSTRACT

Objectives: The burden of nonalcoholic fatty liver disease (NAFLD) is increasing, with an estimated prevalence in Europe of 20–30%. Although most patients present with simple steatosis, some progress to advanced fibrosis, cirrhosis, and hepatocellular carcinoma. Definite diagnosis and staging require liver biopsy, which is not feasible given the high prevalence of NAFLD. As such, several noninvasive tools have been formulated. However, to date, none have been validated in the Portuguese population. The aim of this study was to determine the diagnostic accuracy of the aspartate aminotransferase to platelet ratio (APRI), the BMI, AST/ALT ratio and Diabetes (BARD), the FIB-4 Index (FIB-4), the Hepamet fibrosis score (HFS), and the NAFLD fibrosis score (NFS) in a Portuguese population.

Methods: A retrospective review of liver biopsies from two hospital centers was performed. Patients with NAFLD and no decompensated cirrhosis, liver cancer, or terminal illness were included. APRI, BARD, FIB-4, HFS, and NFS were calculated for each patient.

Results: A total of 121 individuals were included, of which 21.5% had advanced fibrosis (F ≥ 3). There was a moderate or high correlation between most tools. The negative predictive factor (NPV) and area under receiver operating curve (AUROC) were 89.9% and 0.80 for APRI, 91.8% and 0.84 for BARD, 95.7% and 0.88 for FIB-4, 96.4% and 0.88 for HFS, and 93.0% and 0.86 for NFS, respectively.

Conclusion: The tools analyzed had excellent performance (AUROC ≥ 0.80) and were adequate for ruling out advanced fibrosis (NPV ≥ 89.9%) in a Portuguese population. As such, they are adequate for use in clinical practice or as a part of referral and follow-up programs wherever this population is treated.

Abbreviations: APRI – aspartate aminotransferase to platelet ratio, ALT – alanine aminotransferase, AST – aspartate aminotransferase, BARD – BMI, AST/ALT ratio and Diabetes, BMI – body mass index, FIB-4 – FIB-4 index, HCC – hepatocellular carcinoma, HFS – Hepamet fibrosis score, HOMA-IR – homeostatic model assessment for insulin resistance, IQR – interquartile range, MAFLD – metabolic associated fatty liver disease, NAFLD – nonalcoholic fatty liver disease, NASH – nonalcoholic steatohepatitis, NFS – NAFLD fibrosis score, OMIC – genomics, transcriptomics, proteomics, and metabolomics, T2DM – type 2 diabetes mellitus

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Introduction

From a previously thought as benign finding, nonalcoholic fatty liver disease (NAFLD) has risen to a leading cause of chronic liver disease. Currently, its prevalence is estimated at 25% and is expected to rise in parallel with the obesity pandemic [1]. Although there are few studies on the prevalence of NAFLD, and especially of nonalcoholic steatohepatitis (NASH) and advanced fibrosis, in Europe, evidence points to around 20% to 30%, with individual countries' prevalence being directly proportional to the prevalence of type 2 diabetes (T2DM), obesity, and other features of the metabolic syndrome [2].

Although cardiovascular disease is still an important cause of death in NAFLD patients, liver-related mortality is increasing and relates to the stage of liver fibrosis [3,4]. Similarly to alcoholic fatty liver disease, patients can progress to cirrhosis and hepatocellular carcinoma (HCC). In fact, in the United Kingdom, in 2010, NAFLD-associated HCC accounted for more than a third of all liver cancer cases, with a 10-fold increase in the previous 10 years. In the United States of America, it is estimated that the rate of NASH will double from 2015 to 2030 [5].

The definitive diagnosis and staging of NAFLD is dependent on liver biopsy, an invasive procedure with inherent risks [6]. This poses a significant hurdle: with such a high

CONTACT Joana Rigor rigorj.md@gmail.com Internal Medicine Department, Vila Nova de Gaia/Espinho Hospital Centre, Vila Nova de Gaia, Portugal,

prevalence in the general population and with most patients with NAFLD not presenting with advanced fibrosis, selecting patients that benefit from liver biopsy becomes essential [7].

Imaging methods have been increasingly used in the assessment of NAFLD, from more classic ultrasound and Vibration Controlled Transient Elastography (Fibroscan®), to newer magnetic resonance imaging and elastography techniques. While they allow for the evaluation of liver steatosis and high-stage fibrosis, they are still suboptimal in detecting low-stage fibrosis. Moreover, as some of these techniques are still in early stages, their use is dependent on local availability and the costs can be prohibitive at the primary care level [8–10]. Newer serological markers are being investigated as possible direct methods of detecting advanced fibrosis and, consequently, patients at increased risk for cirrhosis, HCC, and other liver complications. These novel markers might be used individually or combined with other clinical data to provide a more accurate diagnosis [11–13]. OMIC (genomics, transcriptomics, proteomics, and metabolomics) approaches might one day offer personalized diagnosis and therapeutic pathways [14].

Over the past two decades, several noninvasive tools have been created or adapted for NAFLD, with the intent of discriminating for advanced fibrosis, thus identifying patients more likely to develop cirrhosis and other liver complications [15]. The most often used and recommended of these tools are the BARD (BMI, AST/ALT ratio, and Diabetes) score, the FIB-4 Index, and the NAFLD fibrosis score (NFS) [16,17]. Older scores are also used, such as the APRI (AST to Platelet Ratio Index) [16]. Most recently, the Hepamet fibrosis score (HFS) was developed from a multicenter population of biopsy proven NAFLD patients [18]. Both APRI and FIB-4 were initially developed in patients with viral hepatitis but have since been validated in patients with NAFLD [19–22]. NFS, BARD, and the HFS were specifically created for the NAFLD population [18,23,24] Relying on clinical and biochemical data that are usually already available, these tools are easy and affordable to use in any region and clinical setting. As such, they have been recommended in all patients with NAFLD to rule-out those with advanced fibrosis (F ≥ 3) who will warrant further staging [16]. While easy to calculate, FIB-4, HFS, and NFS have dual cutoffs, with a middle 'indeterminate' or 'intermediate risk' range, which can pose a barrier to the interpretation of results [18,20,23]. For APRI, several cutoffs have been proposed, most commonly 0.7 and 1.0 [22,25]. To the best of our knowledge, these tools have not yet been validated in a Portuguese population.

The aim of this study was to determine the diagnostic accuracy of APRI, BARD, FIB-4, HFS, and NFS in a Portuguese population of biopsy proven NAFLD patients.

Material and methods

The study was conducted at two hospital centers (Vila Nova de Gaia/Espinho Hospital Center and Trás-os-Montes e Alto Douro Hospital Center), both located in the northern region of Portugal. The main poles are in the cities of Vila Nova de Gaia and Vila Real, respectively, and together they serve a population of over 600,000. The population of Vila Nova de Gaia/Espinho Hospital Center is a mix of urban and rural, while that of Trás-os-Montes e Alto Douro Hospital Center is mostly rural.

We reviewed all liver biopsies performed in adults (age ≥18 years) between 1 January 2007 and 31 December 2018. Clinical and laboratory data from the time of the biopsy was gathered via clinical file review. A diagnosis of NAFLD warranted: 1) exclusion of other etiologies of liver disease, including auto-immune, viral, toxic, metabolic, and genetic, 2) no reference to excessive daily alcohol consumption (>20 g/day for women and >30 g/day for men), 3) no other apparent diagnosis on biopsy. Patients were excluded if they presented with decompensated cirrhosis, cancer, or terminal illness. Clinical data collected included age, gender, body mass index (weight in kg divided by height^2 in m), clinical evidence of decompensated cirrhosis (ascites, encephalopathy, esophageal varices), diagnosis of diabetes mellitus or use of antidiabetic drugs; laboratory levels were obtained for platelets, AST, albumin, fasting glucose and, when available, fasting insulin. Histological data was drawn from liver biopsy reports. Liver histology was assessed by both general gastrointestinal pathologists and specialized hepatologists, according to the availability in each center. Brunt *et al.* staging system was used [26]. Biopsies were not included if size was <15 mm of length.

The APRI, BARD, FIB-4, HFS, and NFS were calculated for each individual, with clinical and laboratorial data extracted to the closest date possible to the biopsy and always under 3 months. The cutoffs analyzed for each tool were ≥ 0.7 and ≥ 1.0 for APRI, ≥ 2 for BARD, ≥ 1.3 and > 2.67 for FIB-4, ≥ 0.12 and > 0.47 for HFS, and ≥ -1.455 and > 0.675 for NFS, as previously determined [18,21–23].

Sample size was calculated assuming α of 0.05, β of 0.2, a negative-to-positive cases ratio of 4 and an area under the receiver operating characteristic curve (AUROC) of 0.75; a sample size of 65 (13 cases in positive group, 52 cases in negative group) was obtained. Descriptive analyses were expressed as mean and standard deviation (SD) for continuous variables with normal distribution, mean and interquartile ranges (IQR) for continuous variables without normal distribution, and absolute number and/or percentages for categorical variables. Student t test for independent samples or Mann-Whitney was used to compare means. Spearman's rank correlation was used to assess the relationship between the various tools, with 0.30–0.49 being a low correlation, 0.50–0.69 a moderate correlation, 0.70–0.89 a high correlation, and ≥ 0.90 a very high correlation. For each tool and cutoff, prognostic accuracy measures were calculated, namely sensitivity, specificity, predictive values, likelihood ratios, and AUROC. For the AUROC, a value of < 0.50 suggested no discrimination, 0.50–0.59 poor discrimination, 0.60–0.69 fair, 0.70–0.79 good, and ≥ 0.80 excellent. A p value of < 0.05 was considered statistically significant. All analyses were conducted using IBM SPSS Statistics for Windows, Version 27.0 (Armonk, NY: IBM Corp).

Table 1. Demographic, clinical, and histological characteristics of the population.

	All (n = 121)	F < 3 (n = 95)	F ≥ 3 (n = 26)
Sex, male (%)	52.1%	56.8%	34.6%
Age (mean ± SD)	49.0 ± 13.5	46.4 ± 13.1***	58.5 ± 10.3***
BMI (mean ± SD)[a]	30.2 ± 4.7	29.5 ± 4.2**	32.5 ± 5.6**
T2DM (%)	34.2%	23.2%***	76.0%***
HOMA-IR [median (IQR)][b]	3.6 (1.9–5.4)	3.4 (1.6–5.0)	4.9 (3.8–11.5)
Platelets, x10^9 (mean ± SD)	207.2 ± 66.4	219.4 ± 59.4***	162.8 ± 72.5***
Albumin (mean ± SD)	4.6 ± 0.4	4.7 ± 0.3***	4.3 ± 0.5***
AST, U/L [median (IQR)]	37.0 (28.0–58.0)	34.0 (27.0–50.0)**	54.0 (36.8–77.3)**
APRI [median (IQR)]	0.5 (0.3–0.5)	0.4 (0.3–0.6)***	0.9 (0.5–1.8)***
BARD [median (IQR)][a]	1.0 (1.0–3.0)	1.0 (0.0–2.0)***	4.0 (2.0–4.0)***
FIB-4 [median (IQR)]	1.1 (0.7–2.4)	0.9 (0.7–1.4)***	3.1 (2.3–4.3)***
HFS [median (IQR)][c]	0.07 (0.02–0.39)	0.04 (0.01–0.21)***	0.53 (0.32–0.84)***
NFS [median (IQR)][a]	−1.6 [(−2.9)-(−0.5)]	−2.0 [(−3.1)-(−0.9)]**	0.8 [(−0.9)-1.4]**
Biopsy length, mm [median (IQR)]	17.0 (15.0-20.0)	17.0 (15.0-20.0)	18.0 (15.0–20.5)
Portal areas, *n* [median (IQR)]	15.5 (11.0-19.0)	16.5 (11.8–20.0)	12.5 (10.3–15.8)
Steatosis grade [median (IQR)]	2.0 (1.0-3.0)	2.0 (1.0-3.0)	1.5 (1.0-3.0)

APRI – aspartate aminotransferase to platelet ratio, AST – aspartate aminotransferase, BARD – BMI, AST/ALT ratio, and Diabetes, BMI – body mass index, FIB-4 – FIB-4 index, HFS – Hepamet fibrosis score, HOMA-IR – homeostatic model assessment for insulin resistance, IQR – interquartile range, NFS – NAFLD fibrosis score; T2DM – type 2 diabetes mellitus; a- sample of 108 due to missing BMI, b- sample of 75 due to missing insulin levels, c- sample of 95 due to missing insulin levels/diabetes status; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 2. Spearman's rank correlation between fibrosis assessment tools.

	BARD	FIB-4	HFS	NFS
APRI	0.32	0.70	0.64	0.48
BARD	-	0.62	0.67	0.70
FIB-4	-	-	0.86	0.86
HFS	-	-	-	0.88

APRI – aspartate aminotransferase to platelet ratio, BARD – BMI, AST/ALT ratio, and Diabetes, FIB-4 – FIB-4 index, HFS – Hepamet fibrosis score, NFS – NAFLD fibrosis score; $p < 0.001$

This study was approved by the institutions' Ethics Committee.

Results

A total of 121 patients were included, all Caucasian and of Portuguese origin, of which 52.1% male, with a mean age of 49.0 ± 13.5 years. Over one-fifth (21.5%) had advanced fibrosis (F ≥ 3); these patients were older (57.9 ± 10.0 vs. 46.5 ± 13.3), had higher BMI (32.5 ± 5.6 vs. 29.5 ± 4.2), higher prevalence of DM (76.0% vs. 23.2%), lower platelet count (162.8 ± 72.5 vs. 219.4 ± 59.4), lower serum albumin (4.3 ± 0.5 vs. 4.7 ± 0.3), and higher AST [54.0 (36.8–77.3) vs. 34.0 (27.0–50.0)] than those without advanced fibrosis. The demographic clinical, and histological characteristics of the population are presented in Table 1.

APRI ≥ 0.7 was found in 26.4%, APRI ≥ 1.0 in 18.2%, BARD ≥ 2.0 in 28.9%, FIB-4 ≥ 1.3 in 42.1%, FIB-4 > 2.67 in 20.7%, HFS ≥ 0.12 in 42.1%, HFS > 0.47 in 23.2%, NFS ≥ -1.455 in 46.7% and NFS > 0.675 in 13.1% of patients. There was a low correlation between APRI and BARD, and APRI and NFS; a moderate correlation between APRI and HFS, BARD and FIB-4, and BARD and HFS; and a high correlation between APRI and FIB-4, BARD and NFS, FIB-4 and HFS, FIB-4 and NFS, and HFS and NFS (Table 2).

Table 3 lists the results of the different diagnostic accuracy methods for each tool and cutoff. Sensitivity ranged from 46.2% to 90.5% and specificity from 61.6% to 97.7%. When selecting the lower cutoff for each tool, NPV was ≥ 89.9%, with HFS having the highest NPV (96.4%). The AUROC was 0.80 for APRI, 0.84 for BARD, 0.88 for FIB-4, 0.88 for HFS, and 0.86 for NFS (Figure 1).

Discussion

To the best of our knowledge, this is the first validation study of the tools here analyzed (APRI, BARD, FIB-4, HFS and NFS) in a Portuguese NAFLD population. Portugal ranks 17th in

Table 3. Diagnostic accuracy methods for each of the fibrosis assessment tools and cutoffs.

	APRI (≥ 0.7)	APRI (≥ 1.0)	BARD	FIB-4 (≥ 1.3)	FIB-4 (> 2.67)	HFS (≥ 0.12)	HFS (> 0.47)	NFS (≥ -1.455)	NFS (> 0.675)
Sensitivity, % (95% CI)	65.4 (44.3–82.8)	46.2 (26.7–66.6)	72.7 (49.8–89.3)	88.5 (69.9–97-6)	61.5 (40.6–79.8)	90.5 (69.6–98.8)	61.9 (38.4–81.9)	81.0 (58.1–94.6)	57.1 (34.0–78.2)
Specificity, % (95% CI)	84.2 (75.3–90.9)	89.5 (81.5–94.8)	77.9 (67.7–86.1)	70.5 (60.3–79.4%)	90.5 (82.8–95.6)	71.6 (60.0–81.5)	87.8 (78.2–94.3)	61.6 (50.5–71.9)	97.7 (91.9–99.7)
LR+ (95% CI)	4.1 (2.4–7.1)	4.4 (2.1–9.0)	3.3 (2.1–5.3)	3.0 (2.1–4.2)	6.5 (3.3–13.0)	3.2 (2.2–4.7)	5.1 (2.5–10.2)	2.1 (1.5–3.0)	24.6 (6.0–101.6)
LR- (95% CI)	0.4 (0.2–0.7)	0.6 (0.4–0.9)	0.4 (0.2–0.7)	0.2 (0.1–0.5)	0.4 (0.3–0.7)	0.1 (0.0–0.5)	0.4 (0.3–0.8)	0.3 (0.1–0.8)	0.4 (0.3–0.7)
PPV, % (95% CI)	53.1 (39.7–66.1)	54.6 (36.9–71.1)	45.7 (34.4–57.5)	45.1 (36.9–54.6)	64.0 (47.1–78.0)	47.5 (38.0–57.1)	59.1 (41.8–74.4)	34.0 (26.9–42.0)	85.7 (59.2–96.1)
NPV, % (95% CI)	89.9 (83.9–93.8)	85.9 (80.9–89.7)	91.8 (84.8–95.7)	95.7 (88.4–98.5)	89.6 (84.0–93.4)	96.4 (87.6–99.0)	89.0 (82.4–93.4)	93.0 (84.4–97.0)	90.3 (85.1–93.9)

APRI – aspartate aminotransferase to platelet ratio, BARD – BMI, AST/ALT ratio, and Diabetes, FIB-4 – FIB-4 index, HFS – Hepamet fibrosis score, LR+ – positive likelihood ratio, LR- – negative likelihood ratio, NFS – NAFLD fibrosis score, NPV – negative predictive value, PPV – positive predictive value.

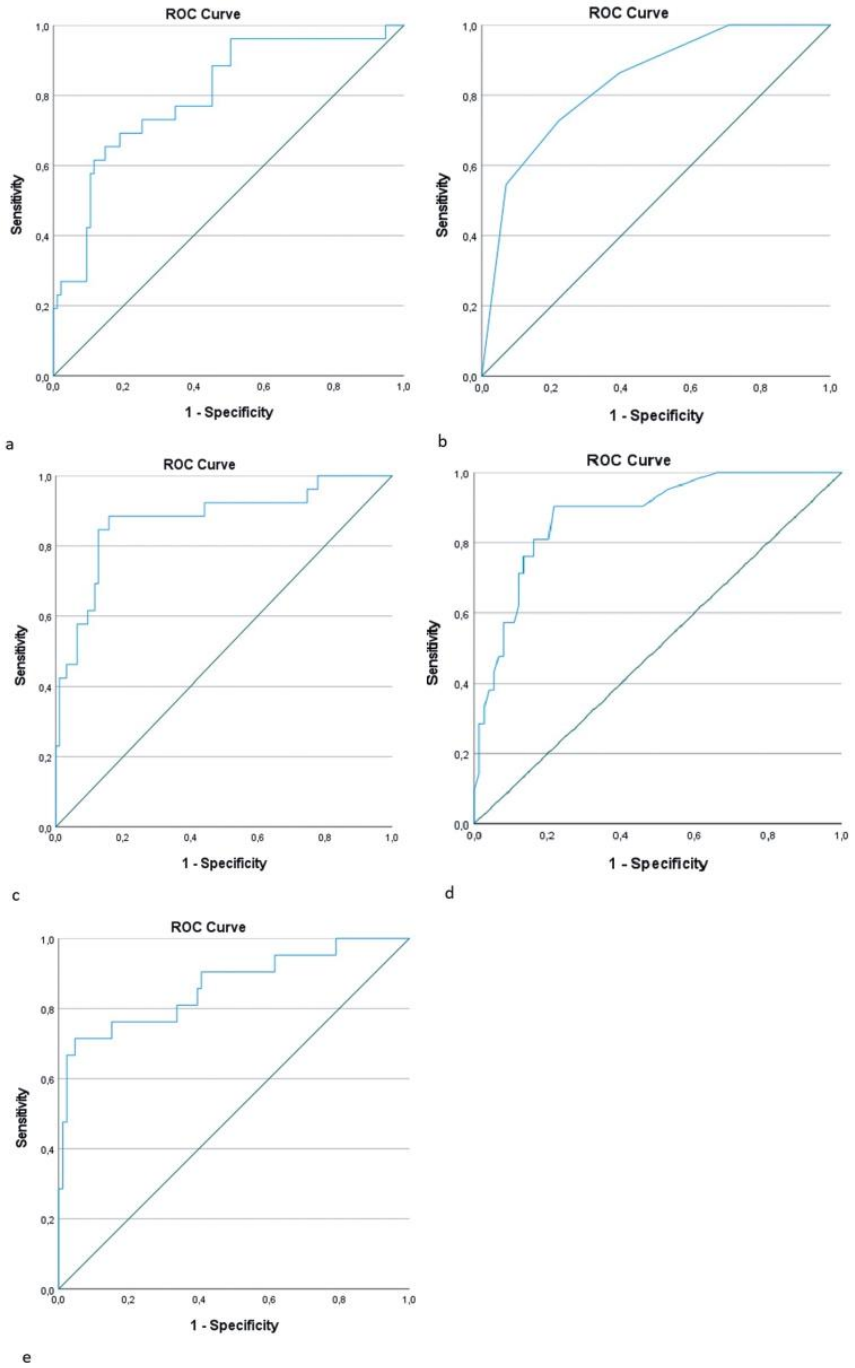

Figure 1. Receiver operating characteristic curve for each of the fibrosis assessment tool (1a – APRI, 1b – BARD, 1c – FIB-4, 1d – HFS, 1e – NFS; APRI – aspartate aminotransferase to platelet ratio, BARD – BMI, AST/ALT ratio and Diabetes, FIB-4 – FIB-4 index, HFS – Hepamet fibrosis score, NFS – NAFLD fibrosis score).

prevalence of obesity in adults and 2nd in prevalence of overweight in 11-year-olds [27], which suggests a high prevalence of NAFLD. This volume of patients can overwhelm health services if an effective severity screening is not applied.

The prevalence of advanced fibrosis in the population studied (21.5%) was within the range (11.1–30%) of what has been described in the literature and in the studies used to develop and validate these tools, which suggests

adequate representation of patients with NAFLD [4,17,18,22–24].

Most tools had a moderate or high correlation with each other. Recommendations usually favor the use of FIB-4 and NFS [16], while HFS is relatively new [18]; these three tools presented the highest correlation between them.

The AUROC for each tool was between 0.80 and 0.88. These values are comparable or superior to what has been described and point to an excellent performance in this setting [16,18,21–24]. As seen in other studies, when the lower cutoffs were select, the NPV were high but the PPV were low [23,24]. This is optimal as the goal of these tools is to exclude advanced fibrosis and help select patients that require additional follow-up and testing, such as liver biopsy [16,28]. FIB-4 and HFS presented the highest AUROC of 0.88, with an NPV of 90.5% for HFS ≥ 0.12 and of 88.5% for FIB-4 ≥ 1.3. However, HFS requires the input of more variables, including insulin levels in non-diabetic patients, a blood test less frequently available [18,20]. As such, and in accordance with other authors [29–32], we recommend FIB-4 ≥ 1.3 as an indicator for the need of further testing or referral to a specialist, individually or as a part of a stepwise algorithm.

The retrospective nature of this study is a methodological limitation. The selection of patients was not randomized as biopsies were performed based on clinical determinants before the inclusion in this study. Also of note, the biopsies were evaluated by more than one pathologist. To mitigate these possible biases, the authors reviewed the reports and clinical data for all biopsies performed in adults in these two centers and in the given time frame.

Another limitation is the population studied, as it included only Caucasian patients of Portuguese origin; this might not reflect the population treated in other areas of the country. The number of patients was relatively small for a validation study and there were also several missing values that further reduced the population. However, in every case, the sample size exceeded the necessary to provide sufficient statistical power.

Recently, a new definition of NAFLD has been proposed, called metabolic associated fatty liver disease (MAFLD), which relies on 'positive criteria' that include excess weight, type 2 diabetes, and other metabolic risk abnormalities [33,34]. In this new context, a recent study has pointed to better performance of FIB-4 and NFS compared to APRI and BARD, but also for the need to develop new fibrosis markers [35]. Moving forward, both new and old fibrosis scores should be integrated in this new concept of MAFLD.

Conclusion

In a Portuguese population, APRI, BARD, FIB-4, HFS, and NFS proved to be reliable tools to rule-out advanced fibrosis in NAFLD patients. Given its high NPV and ease of use, the authors recommend FIB-4 be preferred whenever a single tool is used. As NAFLD is growing in prevalence, it is important to effectively screen patients based on severity, both at an individual clinical level as well as integrated in referral and follow-up programs. The authors hope validation of these tools can provide an evidence-based foundation for the implementation of such practices. The eventual implementation of MAFLD will warrant future reassessment.

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Conflicts of interest

The authors have no relevant conflicts of interest to disclose. Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Data availability statement

The data that support the findings of this study are available from the corresponding author, J.R., upon reasonable request.

ORCID

Joana Rigor http://orcid.org/0000-0002-3386-8055
Andreia Diegues http://orcid.org/0000-0001-6130-7848
José Presa http://orcid.org/0000-0003-0861-4296
Pedro Barata http://orcid.org/0000-0002-4537-5450
Daniela Martins-Mendes http://orcid.org/0000-0001-9709-9948

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5. How sarcopenia, muscle mass, muscle strength, and physical performance relate to non-alcoholic fatty liver disease: a systematic review

5.2. Material and Methods

5.2.1. Protocol and search strategy

The review was registered in the International Prospective Register of Systematic Reviews (PROSPERO), ID CRD42020209051. The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines were followed. (21) A literature search was performed, on October 16th 2020, in PubMed, Web of Science, Scopus and Latin American and Caribbean Health Sciences Literature (LILACS) database with the following query ("Non alcoholic Fatty Liver Disease" [Mesh] OR "NAFLD" OR "Nonalcoholic Fatty Liver Disease" OR (Fatty AND Liver* AND Nonalcoholic) OR (Nonalcoholic AND Steatohepatiti*) OR "NASH") AND (Sarcopenia [Mesh] OR Sarcopen* OR (Loss AND muscle) OR "low muscle mass"); in Scopus and LILACS Mesh terms were not used. Included languages were English, Portuguese, Spanish and French, and search was not restricted by date of publication. Furthermore, the list of references of pertinent articles were examined for relevant studies.

5.2.2. Study selection and eligibility criteria

Studies were eligible if they were analytical studies and included information about association between muscle mass/strength/performance and the presence/development/severity of NAFLD. No other restrictions were imposed, such as population group, sex, or age.

5.2.3. Data extraction

After excluding duplicates, search results were analyzed by two separate researchers (JR and DMM) independently with use of a reference manager (EndNote 20, Clarivate, Philadelphia, PA, 2013). In a first stage, records were screened by title and abstract, and, in a second stage, references' eligibility was assessed by full-text analysis. Disagreements were resolved by conference. Data was extracted by JR and confirmed by DMM in accordance to previously determined variables: author, year of publication, country of origin, type of study, population, sex and age distribution, low muscle mass/strength/performance definition, method of NAFLD diagnosis/severity assessment, association or risk measure and confounder adjustment. The Newcastle-

Ottawa Scale was applied by JR and confirmed by DMM, to assess the risk of bias for each included study.

5.3. Results

Search yielded 894 references and included studies' bibliography review identified 1 additional study. In the end, 53 studies (11 unpublished poster abstracts) were included in the final selection. Figure 1 shows the flowchart for the literature search and selection process.

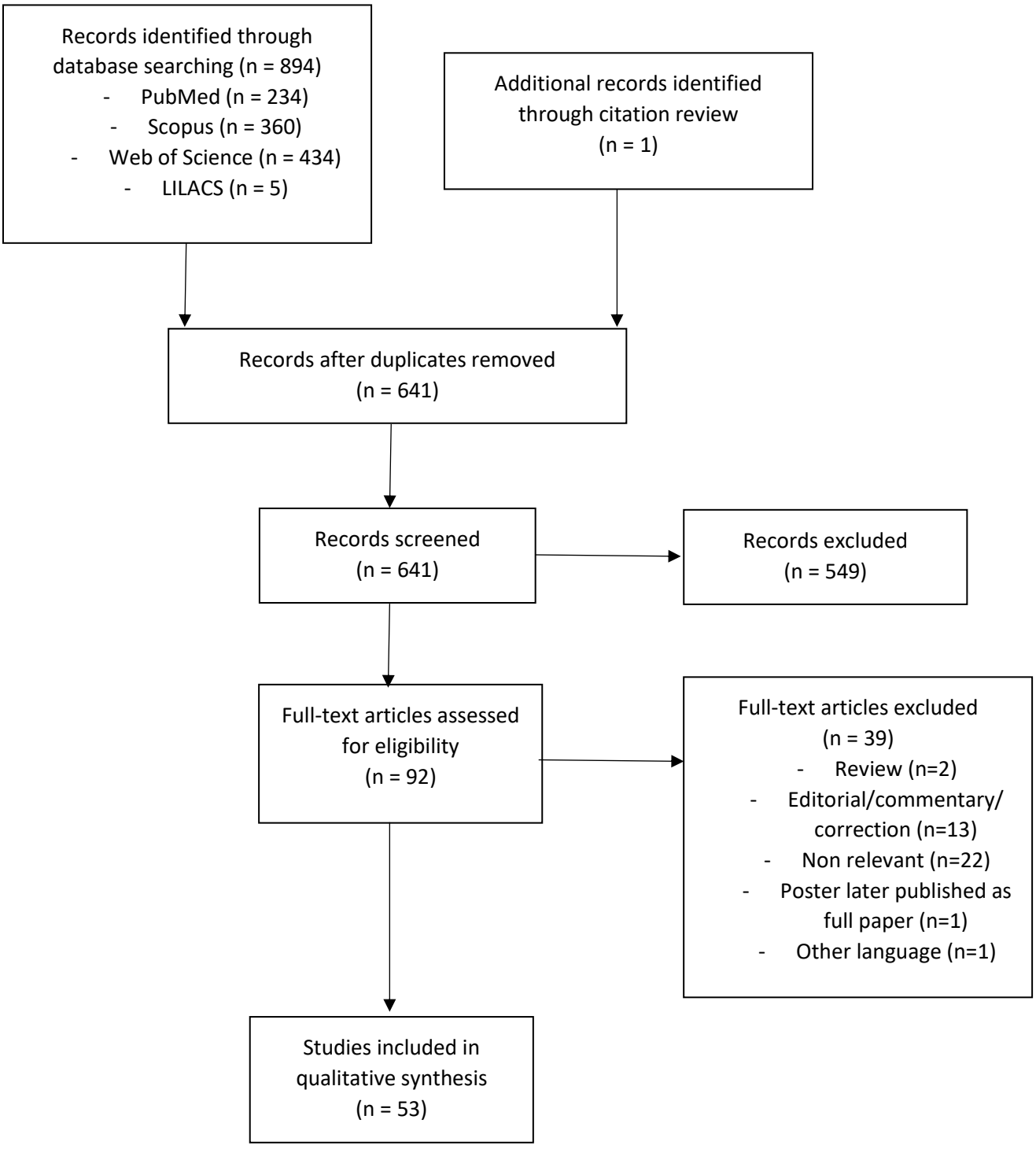

Figure 1 - Flowchart for the literature search and selection process

Table 1 summarizes the characteristics and findings of the studies; tables 2-6 expand on the findings.

The methodology of the studies varied significantly. While liver biopsy (LB) is the gold-standard for NAFLD diagnosis, it was only performed in 11 studies (20.8%).(100-110) Most frequently, authors used imaging techniques(111-137), validated clinical equations(138-150), or controlled attenuation parameter in transient elastography(151, 152). To assess NAFLD severity, LB was used in the greatest number of studies.(101-110) However, when considering sample size, non-invasive scores were the most employed.(111, 122, 137, 140, 143, 145)

Most frequently, studies used ASM or SMM, as opposed to muscle cross area, to determine muscle mass. Weight adjusted measures were the most common,(101-104, 111-124, 138-142, 151, 152) followed by BMI adjusted(102, 105, 111, 112, 117, 125-128, 141, 143-145) and height adjusted measures(105-107, 112-114, 126, 129-131). When weight or BMI adjusted measures were used, there was consistently an inverse association between muscle mass and NAFLD presence or severity. This contrasted with studies with height adjusted measures which either showed a positive(112-114, 126, 131) or no association(106, 107, 114, 129, 130). Six studies used measurements adjusting for fat,(105, 107, 132-134, 139, 150) and all found an inverse relationship with NAFLD, in at least part of the population analyzed.

Muscle strength was primarily assessed by handgrip strength (HGS),(114, 115, 121, 123, 126, 129, 131, 135, 146-149) though elbow flexion strength (EFS)(123, 136) and knee extension strength (KES)(115, 123, 126, 136) were also used. Most studies showed an inverse relationship with NAFLD or NAFLD severity. Physical performance was determined by gait speed in three studies and was not found to be associated with the presence(114) or severity(112) of NAFLD when taken in isolation but was when used for the definition of NAFLD.(131)

Only three studies(112, 114, 131) defined sarcopenia as a compound of low muscle mass and low muscle strength and/or performance, with conflicting results probably attributed to different assessment methods.

Fifteen studies (28.3%) analyzed data by sex categories,(101, 107, 110, 114-117, 127, 132, 136, 138, 147, 149, 151, 152) while four studies only looked at either a male(129, 135) or female(118, 123) population. An association between variables was found in men but not women in five studies(101, 110, 115, 117, 127) and women but not men in two(114, 132); in the first, measures were adjusted for weight or BMI, while in the latter they were adjusted for height or fat mass.

Longitudinal studies were rare but suggested that low muscle mass precedes NAFLD and that variations in muscle mass affect the development and remission of this disease.(105, 107, 113, 116, 134, 141)

The results for the Newcastle-Ottawa Scale are reported in Table 7. Most studies included were retrospective analysis of a selected sample within large surveys of the general population, created to assess overall health and nutritional status and not to respond to this specific research question. There is a possible bias of selection as samples within these surveys were frequently chosen according to the availability of the variables of interest. Adjustment for confounders was not performed or not reported for at least some exposure or outcome of interest in 19 (35.8%) papers. There was seldom information on blinding of the researchers.

Table 1 – Summary of characteristics and main results of studies included

First author (year)	Diagnosis of NAFLD	Assessment of NAFLD severity	Assessment of muscle mass		Assessment of muscle strength and/or performance	Main results
			Method	Parameter		
Tsien, C (2012) [a] (108)	LB	LB	CT	TPA	-	Lower TPA in NASH and NASH cirrhosis than controls or steatosis. Fibrosis and lobular inflammation inversely correlated with TPA.
Choi, YJ (2013) [a] (118)	US	-	BIA	SMM/weight (Q1)	-	Increased AOR of NAFLD.
Moon, JS (2013) (139)	FLI	-	BIA	SMM/weight SMM/VFA (continuous and Q4)	-	Negative correlation, decreased AOR for SMM/VFA.
Hong, HC (2014) (119)	CT	-	DXA	SMM/weight (Q1)	-	Increased AOR of NALFD.
Issa, D (2014) (109)	LB	LB	CT	TPA	-	Lower TPA in NAFLD, and in NASH-cirrhosis vs. NASH.
Lee, YH (2015) (140)	HSI (>36), CNS (≥40), LFS (≥−0.640)	BARD (≥2), FIB-4 (≥2.67)	DXA	ASM/weight (<32.2% ♂, 25.5% ♀)	-	Increased AOR of NAFLD and significant fibrosis.
Yamaguchi, A (2015) [a] (152)	CAP[c]	LSM (≥9.0)	BIA	SMM/weight	-	Lower SMM/weight in patients with LSM ≥9.0
Hashimoto, Y (2015) (151)	CAP (>237.8)	-	DXA	SMM/weight (continuous)	-	Decreased AOR of NAFLD.
Joo, SK (2016) [a] (110)	LB[c]	LB	DXA	ASM/weight	-	Decreasing ASM/weight with increasing fibrosis.
Kim, HY (2016) (138)	FLI (≥60)	-	DXA	ASM/weight (continuous)	-	Increased AOR of NAFLD with lower ASM/weight.
Kim, W (2016) [a] (101)	LB	LB	BIA	ASM/weight (Q1)	-	Lower ASM/weight in NAFLD. Increased AOR of NASH.
Lee, YH (2016) (145)	LFS≥-0.640[c]	NFS (Q4), FIB-4 (≥2.67), Forns index[d] (Q4)	DXA	ASM/BMI	-	Decreased AOR for NFS and FIB-4, NS for Forns index.
Poggiogalle E, (2016) (150)	FLI	-	DXA	TrFM/ASM	-	Positive correlation.
Shen, H (2016) [a] (130)	US	-	BIA	SMM/height^2 (≤10.75% ♂, ≤6.75 ♀)	-	Decreased OR, NS AOR.
Kallwitz, ER (2017) [a] (143)	FLI, NFLS	NFS	NR	ASM/BMI	-	Increased AOR for every 1SD decrease.

Koo, BK (2017) (102)	LB	LB, LSM (NASH and F≥2)	BIA	ASM/weight (<29.0% ♂, <22.9% ♀) ASM/BMI (<0.789 ♂, <0.512 ♀)	-	Increased AOR of NAFLD, NASH, and F≥2.
Osaka, T (2017) (124)	US[c]	LSM	BIA	SMM/weight	-	Inverse correlation. Decreased OR of F≥2.
Peng, TC (2017) (112)	US	US	BIA	SMM/weight (<37.0% ♂, <28% ♀) SMM/height^2 (<10.76 ♂, <6.75 ♀)	Gait speed (<0.8 m/s)	Increased AOR for SMM/weight and SMM/weight + gait speed, NS AOR (but increased OR) for gait speed, decreased AOR for SMM/height.
Petta, S (2017) (104)	LB[c]	LB	BIA	ASM/weight (<37 ♂, <28 ♀)	-	Increase AOR of grade 3 steatosis, ballooning, and fibrosis, but not NASH.
Rachakonda, V (2017) [b] (113)	CT	-	DXA, CT	FFM, FFM/height^2, FFM/weight, MMA, MMA/height^2, MMA/weight	-	Higher FFM, FFM/height^2, MMA, and MMA/height^2. FFM/weight and MMA/weight NS. Resolved vs. persistent NAFLD: NS.
Choe, EK (2018) (125)	US	-	CT	SMA/BMI (<8.37 ♂, 7.47 ♀)	-	Increased AOR of NAFLD.
Choe, EK (2018) (137)	US	FIB-4	Physical examination	WCR (T3)	-	Increased AOR of NAFLD and significant fibrosis.
Kapuria, D (2018) [a] (106)	LB[c]	LB	CT	TPA/height^2	-	Higher TPA/height^2 in advanced steatosis, AOR NS. Fibrosis and NASH NS.
Kim, G (2018) [b] (141)	HSI (>36.0; resolution of NAFLD <30)	-	BIA	ASM/weight ASM/BMI ΔASM/weight ΔASM/BMI	-	Decreased AHR of incident NAFLD and increased AHR of NAFLD resolution.
Kwanten, WJ (2018) [a] (103)	LB	LB	BIA, CT	Muscle mass[c]/weight (<2SD below reference)	-	Low muscle mass more prevalent in NAFLD, and in ≥F2 vs. <F2 vs. NAFL.
Lee, K (2018) (146)	HSI (>36.0)	-	-	-	HGS/BMI (1SD decrease, Q1)	Increased AOR of NAFLD.
Shida, T (2018) (133)	US and elevated ALT[x]	LSM (≥12), CAP (≥260)	BIA	SMM/VFA (Q1)	-	Increased AOR of NAFLD and severity.
Yerragorla, P (2018) [a] (100)	LB	-	CT	SMA	-	Lower SMA in NAFLD.

Zhai, Y (2018) (131)	US	-	DXA	ASM/height^2	HGS (<26 ♂, <18 ♀), Gait speed (<0.8 m/s)	Low muscle mass and low muscle strength and performance (simultaneously) inversely correlated with NAFLD.
Alferink, LJM (2019) (114)	US	LSM (≥8.0kPa)	DXA	ASM/weight, ASM/height^2	HGS, Gait speed	In normal weight ♀: decreased AOR of NAFLD for ASM/weight and ASM/height^2, lower HGS in NAFLD. In ♀ decreased AOR of LSM≥8.0 kPa for ASM/height^2. Remaining NS.
Chen, VL (2019) (115)	CT	-	DXA	ASM/weight	HGS, KES	Negative correlation in ♂, NS in ♀. Lower HGS in NAFLD in ♂, NS in ♀. KES NS.
Chung, GE (2019) (120)	US	US	BIA	ASM/weight (<29% ♂, <22.9% ♀; and Q1)	-	Increased AOR of NAFLD and severity.
Cruz, JF (2019) (136)	US	US	-	-	EFS/BMI, KES/BMI	Inverse relationship with NAFLD. Lower EFS/BMI and KES/BMI in grade 3 steatosis
Debroy, P (2019) (129)	CT	-	DXA	ASM/height^2	HGS/weight (<25th percentile, 25-50th percentile)	ASM/height^2 NS. Low HGS/weight increased AOR of NAFLD.
Gan, D (2019) (121)	US	-	DXA	ASM/weight (<28.64% ♂, <24.12% ♀)	HGS/weight (<51.26% ♂, <35.38% ♀)	Increased AOR of NAFLD.
Gerber, L (2019) [a] (144)	US-FLI	-	DXA	ASM/BMI (<0.789 ♂, <0.512 ♀)	-	Higher prevalence of low ASM/BMI in NAFLD.
Hsing, J C(2019) (142)	FLI (≥60)	-	DXA	ASM/weight (≥29.1 ♂, ≥25.1 ♀)	-	Decreased AOR of NAFLD.
Kang, MK (2019) (111)	US[c]	NFS, FIB-4	BIA	ASM/weight (<29 in ♂, <22.9 in ♀) ASM/BMI (<0.789 in ♂, <0.512 in ♀)	-	Increased AOR of NAFLD.
Kim, B-J (2019) (147)	HSI (per unit increase)	-	-	-	HGS (♂ <28.9, ♀ <16.8)	Increased AOR of NAFLD.
Lee, MJ (2019) [b] (116)	US	-	BIA	ΔASM, ΔASM/weight (T3)	-	Increased AOR for ΔAMS. Higher loss of ASM/weight in NAFLD.
Mizuno, N (2019) [b] (107)	LB[c]	LB, ΔALT (decrease)	BIA	SMM/height^2, SMM/FM		Baseline: lower SMM/FM in NASH than in simple steatosis, SMM/height^2 NS, fibrosis NS. Follow-up: SMM/FM with increased AOR of decrease in ALT.

Oshida, N (2019) (126)	US	-	BIA	ASM/BMI, ASM/height^2	HGS, KES	Lower ASM/BMI, lower KES (in <60y), and higher ASM/height^2 in NAFLD. KES in >60y and HGS NS.
Seko, Y (2019) [b] (105)	LB[c]	LB, ΔALT (decrease >30%)	BIA	ASM/BMI, ASM/FM, ASM/height^2	-	Baseline: higher ASM/BMI and ASM/FM in F<2 and NAS<6, NS for ASMI/height^2. Follow-up: ΔASM/FM increased AOR of ALT decreased, ΔASM/BMI NS.
Seo, DH (2019) (117)	US	US	BIA	ASM/weight (<29.0% ♂, <22.9% ♀) ASM/BMI (<0.789 ♂, <0.512 ♀)	-	Increased AOR in ♂, NS in ♀. Higher proportion of moderate-to-severe NALFD in low ASM/weight.
Shida, T [b] (134)	US[c]	LSM, CAP	BIA	ΔSMM/VFA	-	Decreased CAP in improved SMM/VFA, ΔLSM NS.
Su, X (2019) (132)	US	-	BIA	ASM/VFA (T1)	-	♂ increased OR (not AOR), ♀ increased AOR of NAFLD.
Wijarnpreecha, K (2019) (122)	US	NFS (>0.676 or >0.12 if ≥65y)	BIA	SMM/weight (<37.0% in ♂, <28.0% in ♀)	-	Increased AOR of NAFLD and significant fibrosis.
Zhang, Y (2019) (123)	^{1}H MRS	-	DXA	ASM/weight SMM/weight (continuous)	HGS/weight, KES/weight, EFS/weight	Negative correlation for all except for EFS/weight (NS).
Hao, L (2020) (135)	US	-	-	-	HGS/weight	Decreased AOR of NAFLD
Hyun Kim, K (2020) (128)	US	CAP, LSM	BIA	ASM/BMI (<0.789 ♂, <0.521 ♀)	-	Higher prevalence of low ASM/BMI in NAFLD vs. CHB. Higher LSM in low ASM/BMI. CAP NS.
Kang, S (2020) (148)	HSI (>36.0)	-	-	-	HGS/BMI (Q1)	Increased AOR of NAFLD.
Park, SH (2020) (149)	LFS (>-0.640)	FIB-4, NFS	-	-	HGS/BMI (Q4)	Decreased AOR for NAFLD. HGS/BMI quartiles showed inverse relationships with FIB-4 and NFS score quartiles.
Tanaka, M (2020) (127)	US	-	CT	SMA/BMI	-	Decreased AOR of NAFLD in ♂, NS in ♀.

[a] poster, [b] longitudinal, [c] for population definition only; ♂– male, ♀– female, ^{1}H-MRS – single-voxel proton magnetic resonance spectroscopy, AHR – adjusted hazard ratio, ALT – alanine aminotransferase, AOR – adjusted odds ratio, ASM – appendicular skeletal muscle mass (kg), BIA – bioelectrical impedance analysis, BMI – body mass index (kg/m^2), CAP – controlled attenuation parameter, CHB – chronic hepatitis B, CNS – comprehensive NAFLD score, CT - computed tomography, DXA – dual-energy X-ray absorptiometry, EFS – elbow flexor strength, FFM – fat-free mass (kg), FIB-4 – fibrosis-4 index, FLI – Fatty Liver Index, FM – fat mass (kg), HGS – handgrip strength (kg), HIV – human immunodeficiency viruses, HSI – Hepatic Steatosis Index, KES – knee extension strength, LB – liver biopsy, LFS – liver fat score, LSM – liver stiffness measurement, MMA – midthigh muscle area (cm^2), NAFLD – non-alcoholic fatty liver disease, NAS – NAFLD activity score, NASH – non-alcoholic steatohepatitis, NFLS – NAFLD Liver Fat Score, NFS – NAFLD fibrosis score, NS – non-significant, OR – odds ratio, Q1 – lowest quartile, Q4 – highest quartile, SD – standard deviation, SMA – skeletal muscle area, SMM – skeletal muscle mass, T1 – lowest tercile, T3 – highest tercile, TPA – total psoas muscle area (cm^2), TrFM – truncal fat mass (kg), US – ultrasound, US-FLI – U.S. Fatty liver index, VFA – visceral fat area (cm^2), WCR – waist-to-calf ratio

Table 2 – Characteristics and results of studies assessing the association of muscle mass and the presence of NAFLD

First author (year)	Country	Study type	Setting and population (size)	Male (%)	Age (y), mean ± SD or median (IQR) or % by age group	Diagnosis of NAFLD	Assesment of muscle mass		Association or risk measure	Confounder adjustment
							Method	Parameter		
Tsien, C (2012) (108)	United States	Cross-sectional	Patient with NAFLD on LB and controls (n=131)	NR	NR	LB	CT	TPA	Non-NAFLD (n=57) vs. NAFLD [a] (n=74): 29.4±7.5 vs. 26.7±8.9	NA
Choi, YJ (2013) (118)	South Korea	Cross-sectional	Women with T2DM (n=1926)	NA	58±12	US	BIA	SMM/weight (Q1)	AOR 2.25 (1.66 - 3.04)	age, HbA1C, WC, TG, SBP and HDL-c
Moon, JS (2013) (139)	South Korea	Cross-sectional	Routine health evaluation (n=9565)	55.3%	46.8±10.6[a]	FLI	BIA	SMM/weight, SMM/VFA	SMM/weight: FLI <20 (n=2821) 43.2±3.9, FLI 20-59 (n=4896) 40.2±4.0, FLI ≥60 (n=1848) 38.2±4.4; p<0.001 SMM/weight: r=-0.56, p<0.001 SMM/VFA: r=-0.41, p<0.001 SMM/VFA Q4: AOR 0.037 (0.029-0.049)	Age, sex, TC, LDL-c, DM, HT, hsCRP
Hong, HC (2014) (119)	South Korea	Cross-sectional	Survey of healthy volunteers, ≥20y (Korean Sarcopenic Obesity Study) (n=452)	36.9%	Sarcopenic: 60 (52-67) Non-sarcopenic: 51 (38-61)	CT	DXA	SMM/weight (Q1)	OR 5.88 (2.33-14.84), AOR 5.16 (1.63-16.33)	age, sex, smoking, physical activity, HOMA-IR, hsCRP, 25[OH]D levels
Issa, D (2014) (109)	United States	Cross-sectional	Patients with NASH on LB and controls (n=75)	NR	NR	LB	CT (L4)	TPA	Non-NAFLD (n=25) vs. NAFLD[a] (n=50): 29.3±0.88 vs. 22.05±2.9	NR
Lee, YH (2015) (140)	South Korea	Cross-sectional	Nationally representative survey (KNHANES 2008-2011), ≥20y (n=15132)	37.1%	50.6±16.6[a]	HSI (>36), CNS[b] (≥ 40), LFS (≥−0.640)	DXA	ASM/weight (<32.2% ♂, 25.5% ♀)	HSI: AOR 1.18 (1.03-1.34) CNS: AOR 1.19 (1.02-1.39) LFS: AOR 1.22 (1.09-1.36)	age, sex, regular exercise, HOMA-IR, smoking, and HT

Hashimoto, Y (2015) (151)	Japan	Cross-sectional	Patients with T2DM (n=145)	54.5%	65.5±11.6[a]	CAP (>237.8)	DXA	SMM/weight	per incremental 1% SMM/weight: ♂ AOR 0.80 (0.64-0.97), ♀ AOR 0.97 (0.81-1.14)	age, BMI, smoking, TG/HDL-c ratio, HbA1c, GGT
Kim, HY (2016) (138)	South Korea	Cross-sectional	Nationally representative survey (KNHANES 2010-2011), ≥19y (n=3739)	31.7%	45.2±2.6[a]	FLI (≥60)	DXA	ASM/weight	♂ OR 1.49 (1.38-1.61), AOR 1.35 (1.17-1.54) ♀ OR 1.47 (1.35-1.60), AOR 1.36 (1.18-1.55)	age, smoking, alcohol drinking, regular exercise, WBC, HOMA-IR, 25[OH]D, number of metabolic syndrome components, DM, HT; and total energy intake, carbohydrate intake. fat intake (energy %) in ♀
Kim, W (2016) (101)	South Korea	Cross-sectional	Patients with NAFLD on LB and controls (n=229)	52.0%	NR	LB	BIA	ASM/weight	NAFLD (n=179) vs. non-NAFLD (n=50): lower, ♂ p=0.002, ♀ p<0.001	
Poggiogalle E, (2016) (150)	Italy	Cross-sectional	Patients with obesity, 18-65y (n=420)	19.0%	45.70±13.9[a]	FLI	DXA	TrFM/ASM	r = 0.221, p <0.001	age, BMI, total FM, FFM, truncal FM, ISI
Shen, H (2016) (130)	United States	Cross-sectional	Nationally representative survey (NHANES 1988-1994), 20-74y (n=9985)	NR	NR	US	BIA	SMM/height^2 (≤10.75% ♂, ≤6.75 ♀)	OR 0.73 (0.66-0.81), AOR 1.00 (0.79 - 1.27)	NR

Kallwitz, ER (2017) (143)	United States	Cross-sectional	Nationally representitive survey (NHANES 1999-2014) (n=7183)	Sarcopenic: 88.6% Non-sarcopenic: 90.7%	Sarcopenic: 56.7±0.84 Non-sarcopenic: 43.02±0.38	FLI, NFLS	NR	ASM/BMI	Every 1SD decrease FLI: AOR 4.34 (3.48-5.41); LFS: AOR 4.56 (3.40-6.12)	NR
Koo, BK (2017) (102)	South Korea	Cross-sectional	Patients with radiologic evidence of hepatic steatosis, ≥18y (Boramae NAFLD registry) (n=309)	46.9%	53±14	LB	BIA	ASM/weight (<29.0% ♂, <22.9% ♀) ASM/BMI (<0.789 ♂, <0.512 ♀)	ASM/weight: OR 3.82 (1.58-9.25), AOR 1.53 (0.50-4.65) ASM/BMI: OR 2.76 (1.13-6.75), AOR 1.27 (0.41-3.95)	age, sex, BMI, smoking, HT, DM, TC, TG, HDL-c, ALT, hsCRP and HOMA-IR
Peng, TC (2017) (112)	United States	Cross-sectional	Nationally representative survey (NHANES 1988-1994), 60-75y (n=2551)	48.6%	66.71 (mean)	US	BIA	SMM/weight (<37.0% ♂, <28% ♀) SMM/height^2 (<10.76 ♂, <6.75 ♀:	SMM/weight: mild steatosis OR 1.33 (1.05-1.69), AOR 1.41 (1.09-1.83); moderate steatosis OR 2.15 (1.71-2.69), AOR 2.22 (1.74-1.83); severe steatosis OR 2.33 (1.73-3.14), AOR 2.30 (1.67-3.17) SMM/height^2: mild steatosis OR 0.74 (0.58-0.93), AOR 0.63 (0.48-0.83); moderate steatosis OR 0.58 (0.47-0.71), AOR 0.52 (0.41-0.67); severe steatosis OR 0.49 (0.37-0.64), AOR 0.44 (0.32-0.61)	age, sex, race/ethnicity, TC, 25[OH]D, HbA1c, CRP, UA, physical activity, smoking
Rachakonda, V (2017) (113)	United States	Longitudinal	Patients with obesity class II or III (RENEW clinical trial) (n=129; undergoing lifestyle intervention 52)	11.6%	47.6 (41.7-52.0)	CT	DXA (or air displacement plethysmography if body weight >136 kg) CT	FFM FFM/height^2 FFM/weight MMA MMA/height^2 MMA/weight	NAFLD (n=58) vs. non-NAFLD (n=71) at baseline FFM: 61.7 (58.8-64.5) vs. 54.5 (53.0-56.0), p<0.001; FFM/height^2: 22.2 (21.6-22.8) vs. 20.2 (19.8-20.6), p<0.01; FFM/weight (%): 49.1 (47.7-50.4) vs. 48.4 (47.4-49.4);), p=0.420; MMA: 149.3 (140.7-158.0) vs. 131.5 (126.2-136.8), p=0.001; MMA/height^2 53.3 (51.1-55.5) vs. 48.8 (47.0-50.6), p=0.002; MMA/weight 1.2 (1.1-1.2) vs. 1.2 (1.1-1.2), p=0.833	NA

									resolved NAFLD (n=20) vs. persistent NAFLD (n=32): NS	
Choe, EK (2018a) (125)	South Korea	Cross-sectional	Routine health evaluation (n=1828)	61.3%	54.9±9.5	US	CT (L3)	SMA (cm^2)/BMI (<8.37 ♂, 7.47 ♀)	AOR 1.51 (1.15-1.99)	age, sex, WC, SBP, FPG, TG, HDL-c, smoking
Choe, EK (2018b) (137)	South Korea	Cross-sectional	Patients with T2DM (n=5507)	50.9%	56.8±10.8	US	Physical examination	WCR (T3)	AOR 1.56 (1.31-1.86)	age, sex, BMI, HT, duration of DM, exercise status, smoking and alcohol history, HbA1c, TC, TG, SITT, medication history for DM and dyslipidemia
Kim, G (2018) (141)	South Korea	Longitudinal	Routine health evaluation, ≥ 20y, (n=15567: non-NAFLD 12624, non-NAFLD and BIA at follow-up 10534, NAFLD 2943)	54.7%	51.4±8.3	HSI (>36.0; resolution of NAFLD <30)	BIA	ASM/weight ASM/BMI	NAFLD at follow-up ASM/weight: T3 AHR 0.44 (0.38-0.51); per percent increase: AHR 0.86 (0.83-0.88) ASM/BMI: T3 AHR 0.47 (0.42-0.54) ΔASM/weight: T3 AHR 0.69 (0.59-0.82); per percent increase: AHR 0.84 (0.79-0.90) ΔASM/BMI: T3 AHR 0.77 (0.65-0.90) Resolution of NAFLD ASM/weight: AHR 2.09 (1.02- 4.28); per percent increase AHR 1.25 (1.10-1.42) ASM/BMI: AHR 2.50 (1.39-4.49) ΔASM/weight: AHR 4.17 (1.90-6.17); per percent increase AHR 1.99 (1.53-2.59)	age, sex, WC, DM, HT, smoking, exercise; and baseline ASM/weight for ΔASM/weight; and baseline ASM/BMI for ΔASM/BMI

									ΔASM/BMI: AHR 3.04 (1.46-6.37)	
Kwanten, WJ (2018) (103)	Belgium	Cross-sectionsectional	Obese patients (n=196)	NR	NR	LB	BIA CT	Muscle mass[c]/weight (<2SD below reference)	NAFLD (n=162) vs. non-NAFLD (n=34): 32.4% vs. 25.9%	NA
Yerragorla, P (2018) (100)	United States	Cross-sectional	Patients with NAFLD on LB and controls (n=166)	48.8%	47±13	LB	CT (L3)	SMA (mm^2)	NAFLD (n=83) vs. controls (n=83) Psoas: right 616±294 vs. 858±257, left 643±299 vs. 835±277 Paraspinal: right 3260±931 vs. 4030±865, left 3318±925 vs. 3927±820 p<0.001 for all	NA
Alferink, LJM (2019) (114)	The Netherlands	Cross-sectional	Health survey, european, ≥45y (The Rotterdam Study) (n=4609)	43.0%	69.3±9.2	US	DXA	ASM/weight ASM/$height^2$	ASM/$height^2$ Normal weight: ♂ AOR 0.63 (0.39-1.02), ♀ AOR 0.48 (0.29-0.80); Overweight: ♂ AOR 0.92 (0.76–1.12), ♀ AOR 1.08 (0.87–1.33) ASM/weight Normal weight: ♂ AOR 0.90 (0.80-1.01), ♀ AOR 0.84 (0.75-0.95); Overweight: ♂ AOR 0.97 (0.92–1.03), ♀ AOR 1.00 (0.94–1.06)	age, study cohorts, weight, height, HOMA-IR, TG, AGR
Chen, VL (2019) (115)	United States	Cross-sectional	Health survey (Framingham Heart Study Offspring and Generation 3 subcohorts) (n=2249)	48.6%	58.5±11.8	CT	DXA	ASM/weight	♂ β −0.0106, p<0.05; ♀ β −0.0038, NS NAFLD vs. non-NAFLD: 26.1 vs. 27.4%, p<0.0001	Age, age^2, physical activity index, cohort, central fat index, lower extremity fat index, muscle steatosis
Chung, GE (2019) (120)	South Korea	Cross-sectional	Routine health evaluation (n=5989)	57.3%	53.2±9.4	US	BIA	ASM/weight	ASM/weight <29 ♂, <22.9 ♀: AOR 1.37 (1.02–1.85) ASM/weight Q1: AOR 1.29 (1.21-1.38)	age, sex, smoking, VFA, HT, DM, TC, LDL-c, HDL-c, TG

Debroy, P (2019) (129)	Italy	Cross-sectional	Men living with HIV (Modena HIV Metabolic Cohort) (n=169)	NA	56.8±5.9	CT	DXA	ASM/height^2	NAFLD (n=57) vs. non-NAFLD (n=112): 7.72±1.22 vs. 8.01±0.81, NS	NA
Gan, D (2019) (121)	China	Cross-sectional	Health survey, 18-80y (Lanxi cohort) (n=3536)	28.7%	52.8±13.1[a]	US	DXA	ASM/weight (<28.64% ♂, <24.12% ♀)	AOR 2.57 (2.03-3.25)	HGS/weight, age, sex, residence area, smoking, physical activity, height, ALT, TG, LDL-c, TC, HbA1c, UA, HT, DM, hsCRP, HOMA-IR, current medications
Gerber, L (2019) (144)	United States	Cross-sectional	Nationally representative surveys (NHANES 1996-2006), ≥20y (n=6416)	48.5%	45.3 (0.4)	US-FLI	DXA	ASM/BMI (<0.789 ♂, <0.512 ♀)	NAFLD (n=1972) vs. non-NAFLD (n=4444): 17.0% vs. 7.1%, p<0.001 After adjustment, p<0.05	age, sex, and race
Hsing, JC (2019) (142)	China	Cross-sectional	Health survey (WELL China cohort), 18-80y, 2 districts (n=3589)	29.3%	<50 y: 41.3%, 50-65 y: 37.9%, >65 y: 20.8%	FLI (≥60)	DXA	ASM/weight (≥29.1% ♂, ≥25.1% ♀)	OR 0.2 (0.1-0.2), AOR 0.1 (0.07-0.13)	age, sex, income, smoking, ALT, HOMA-IR, AFR
Lee, MJ (2019) (116)	South Korea	Longitudinal	Routine health evaluations, ≥ 18y, without NAFLD at baseline, 10-year follow-up (n=4398)	49.4%	46.3±8.3	US	BIA	ΔASM ΔASM/weight (T3)	NAFLD (n=591) vs. non-NAFLD (n=3807): ΔASM/weight −2.24 (−3.51-−0.97) vs. −1.07 (−2.44-0.32) ΔASM ♀ OR 1.67 (1.18-2.36), AOR 2.10 (1.38-3.18); ♂ OR 0.80 (0.60-1.05), AOR 1.61 (1.15-2.26); non-obese: OR 0.95 (0.75-1.22), AOR 1.81 (1.34-2.45); obese: OR 0.96 (0.63-1.47), AOR 1.91 (1.11-3.31)	age, smoking, DM, HT, use of lipidi-lowering drugs, ΔBMI, ΔWC, ΔSBP, ΔHbA1c, ΔTG, ΔLDL-c, ΔHDL-c, ΔAST, ΔALT,

										ΔGGT, ΔUA, ΔTSH and ΔFT4
Oshida, N (2019) (126)	Japan	Cross-sectional	Outpatients followed for lifestyle-related liver diseases (n=253)	46.6%	<30y: 21.7%, 31-60y: 41.9%, >60y: 36.4%	US	BIA	ASM/BMI ASM/height^2	NAFLD (n=153) vs. non-NAFLD (n=100) ASM/BMI: <31y 0.68 vs. 0.93, 31-60y 0.76 vs. 0.85, >60y 0.70 vs. 0.78, p<0.01 ASM/height^2: <31y 21.8 vs. 19.5, 31-60y 21.7 vs. 17.8, >60 18.1 vs. 16.2, p<0.01;	NA
Seo, DH (2019) (117)	South Korea	Cross-sectional	Patients with MetS, 30-64y (Seoul Metabolic Syndrome Cohort) (n=4210)	51.3%	57.4±10.8	US	BIA	ASM/weight (<29.0% ♂, <22.9% ♀) ASM/BMI (<0.789 ♂, <0.512 ♀)	ASM/weight: ♂ AOR 1.58 (1.15-2.17), ♀ AOR 0.97 (0.71-1.38) ASM/BMI: ♂ AOR 1.41 (1.02-1.94), ♀ AOR 1.06 (0.75-1.52)	age, BMI, WC, SBP and DBP, HbA1c, TG (log scale), HDL-c, hsCRP, SITT, use of sulphonylurea, thiazolidinedione, insulin
Su, X (2019) (132)	China	Cross-sectional	Patients with T2DM, 40-75y (n=445)	53.0%	59.4±9.5[a]	US	BIA	ASM/VFA (T1)	♂ OR 4.27(2.12-8.61), AOR 2.83 (0.55-8.43), ♀ OR 3.43 (1.70-6.91), AOR 3.43 (1.41-8.74)	age, DM duration, BMI, WC, SBP, DBP, HbA1c, smoking, alcohol, ALT, AST, TC, TG, HDL-c, LDL-c, medication for DM and dyslipidemia
Wijarnpreecha, K (2019) (122)	United States	Cross-sectional	Nationally representative surveys (NHANES 1988-1994), 20-74y (n=11325)	47.1%	42.7 (mean)	US	BIA	SMM/weight (<37.0% in ♂, <28.0% in ♀)	Total population: OR 2.31 (2.01-2.64), AOR 1.24 (1.04–1.48) Fasting participants (n=5591): OR 2.29 (1.86-2.83), AOR 1.21 (0.95-1.54)	age, sex, ethnicity, BMI, economic status, DM, smoking, HT, TC,

										antihyperlipidemia medication, sedentary physical activity, 25[OH]D deficiency, CRP; and HOMA-IR in fasting participants
Zhang, Y (2019) (123)	China	Cross-sectional	Post-menopausal women in an outpatient clinic, 50–65y (n=96)	NA	59.7±3.6[a]	^{1}H MRS	DXA	ASM/weight SMM/weight	ASM/weight: r=-0.42, p=0.009; SMM/weight: r=-0.28, p<0.001	HOMA-IR
Hyun Kim, K (2020) (128)	South Korea	Cross-sectional	Patients with CLD (n=2168)	61.3%	54.4±12.7	US	BIA	ASM/BMI (<0.789 ♂, <0.521 ♀)	NAFLD (n=957) vs. CHB (n=911): 12.9 vs. 6.6%	NA
Tanaka, M (2020) (127)	Japan	Cross-sectional	Routine health evaluation (Nishimura Health Survey) (n=632)	55.85%	50.6±11.1[a]	US	CT (L3)	SMA (cm^2)/BMI	Per 1.0 $cm^2/kg/m^2$ increase: ♂ AOR 0.59 (0.38-0.89), ♀ AOR 0.50 (0.20-1.24)	age, smoking, exercise, ALT, GGT, TG, HDL-c, SBP, FPG, VFA, and medication for HT, dyslipidaemia and DM

[a]calculated by authors, [b]uric acid was not used due to unavailable data, [c]not specified if ASM or SMM; ♂– male, ♀– female, ^{1}H-MRS – single-voxel proton magnetic resonance spectroscopy, 25[OH]D – 25-hydroxyvitamin D, AFR – android fat ratio, AGR – android-fat-to-ginoid-fat ratio, AHR – adjusted hazard ratio, ALT – alanine aminotransferase, AOR – adjusted odds ratio, ASM – appendicular skeletal muscle mass (kg), AST – aspartate aminotransferase, BIA – bioelectrical impedance analysis, BMI – body mass index (kg/m^2), CAP – controlled attenuation parameter, CHB – chronic hepatitis B, CLD – chronic liver disease, CNS – comprehensive NAFLD score, CRP - C-reactive protein, CT - computed tomography, DBP – diastolic blood pressure, DM – diabetes mellitus, DXA – dual-energy X-ray absorptiometry, FFM – fat-free mass (kg), FLI – Fatty Liver Index, FM – fat mass (kg), FPG – fasting plasma glucose, FT4 – free thyroxine, GGT – gamma-glutamyltransferase, HbA1c – glycated hemoglobin, HDL-c – high-density lipoprotein cholesterol, HGS – handgrip strength, HIV – human immunodeficiency viruses, HOMA-IR – homeostasis model of insulin resistance, hsCRP – high sensitivity C-reactive protein, HSI – Hepatic Steatosis Index, HT – hypertension, IQR – interquartile range, ISI – insulin sensitivity index, KNHANES – Korean National Health and Nutrition Examination Survey, L3 – third lumbar vertebrae, LB – liver biopsy, LDL-c – low-density lipoprotein cholesterol, LFS – liver fat score, MMA – midthigh muscle area (cm^2), MetS – metabolic syndrome NA – not applicable, NAFLD – non-alcoholic fatty liver disease, NFLS – NAFLD Liver Fat Score, NHANES – National Health and Nutrition Examination Survey, NR – not reported, NS – non-significant, OR – odds ratio, Q1 – lowest quartile, Q4 – highest quartile, SBP – systolic blood pressure, SD – standard deviation, SITT – short insulin tolerance test, SMA – skeletal muscle area, SMM – skeletal muscle mass, T1 – lowest tercile, T2DM – type 2 diabetes mellitus, T3 – highest tercile, TC – total cholesterol, TG – triglycerides, TPA – total psoas muscle area (cm^2), TrFM – truncal fat mass (kg), TSH – thyroid-stimulating hormone, UA – uric acid, US – ultrasound, US-FLI – U.S. Fatty liver index, VFA – visceral fat area (cm^2), WBC – white cell blood count, WC – waist circumference, WCR – waist-to-calf ratio, y – years

Table 3 – Characteristics and results of studies assessing the association of muscle strength and/or performance and the presence of NAFLD

First author (year)	Country	Study type	Setting and population (size)	Male (%)	Age (y), mean±SD or median (IQR) or % by age group	Diagnosis of NAFLD	Assesment of muscle strength or performance		Association or risk measure	Confounder adjustment
							Method	Parameter		
Peng, TC (2017) (112)	United States	Cross-sectional	Nationally representative survey (NHANES 1988-1994), 60-75y (n=2551)	48.6%	66.71 (mean)	US	Physical examination	Gait speed (<0.8)	Mild steatosis OR 1.29 (1.01-1.65), AOR 1.12 (0.86-1.45); moderate steatosis OR 1.32 (1.07-1.64), AOR 1.17 (0.92-1.47); severe OR 1.15 (0.88-1.50), AOR 0.94 (0.70-1.25)	age, sex, race/ethnicity, TC, 25[OH]D, HbA1c, CRP, UA, physical activity, smoking
Lee, K (2018) (146)	South Korea	Cross-sectional	Nationally representative surveys (KNHANES 2014-2015), 19-80y (n=8001)	44.5%	49.9±16.4	HSI (>36.0)	Dynamometer	HGS/BMI	1SD decrease: AOR 1.47 (1.35–1.60) Q1: AOR 2.43 (2.05–2.88)	age, sex, education, physical activity, alcohol use, smoking, treatment of illness (CVD, DM, HT, dyslipidemia, cirrhosis, or arthritis), BMI, MetS
Alferink, LJM (2019) (114)	The Netherlands	Cross-sectional	Health survey, european, ≥45y (The Rotterdam Study) (n=4609)	43.0%	69.3±9.2	US	DXA	HGS Gait speed	NAFLD (n=1623) vs non-NAFLD (n=2986) HGS: normal weight ♀ 20.9±5.1 vs. 21.8±6.0 p=0.176, ♂ 33.0±9.2 vs. 35.4±8.5 p=0.036; overweight: ♀ 21.61±5.66 vs. 21.77±5.75 p=0.553, ♂ 36.7±9.1 vs. 36.8±8.9) p=0.841 Gait speed: normal weight ♀ 1.21 (1.08, 1.31) vs. 1.24 (1.11, 1.36) p=0.994, ♂ 1.27 (1.13, 1.38) vs. 1.26 (1.11, 1.40) p=0.727; overweight: ♀ 1.15 (1.02, 1.27) vs. 1.17 (1.03, 1.28)	age, study cohorts, weight, height, HOMA-IR, TG, AGR

									p=0.570, ♂ 1.23 (1.09, 1.34) vs. 1.24 (1.09, 1.35) p=0.990	
Chen, VL (2019) (115)	United States	Cross-sectional	Health survey (Framingham Heart Study Offspring and Generation 3 subcohorts) (n=2249)	48.6%	58.5±11.8	CT	Dynano meter	HGS KES	NAFLD (n=1613) vs. non-NAFLD (n=636) HGS: ♂ 44.5±9.6 vs. 45.8±9.2, p=0.032; ♀ 26.0±6.6 vs. 26.5±6.0, p=0.25 KES: ♂ 28.1±9.0 vs. 28.3±8.5, p=0.68; ♀ 21.9±7.6 vs. 23.1±7.6, p=0.25	NA
Cruz, JF (2019) (136)	Brazil	Cross-sectional	Patients with US (n=102)	36.3%	45.3±13.1	US	Dynamo meter	EFS/BMI KES/BMI	Inverse relationship EFS/BMI: p=0.009 KES/BMI: p=0.006	Age, sex
Debroy, P (2019) (129)	Italy	Cross-sectional	Men living with HIV (Modena HIV Metabolic Cohort) (n=169)	NA	56.8±5.9	CT	Dynamo meter	HGS/weight	<25th percentile: AOR 2.47 (1.01-6.19), 25-50th percentile: AOR 3.05 (1.27-7.61)	age, height, metabolic syndrome, nadir CD4+, intensive smoking, moderate smoking, exposure do PI, NRTI, NNRTI, INSTI
Gan, D (2019) (121)	China	Cross-sectional	Health survey, 18-80y from Lanxi city, Zhejiang Province, China (Lanxi cohort) (n=3536)	28.7%	52.8±13.1[a]	US	Dynamo meter	HGS/weight (<51.26% ♂, <35.38% ♀)	AOR 1.47 (1.21-1.80)	ASM/weight, age, sex, residence area, smoking, physical activity, height, ALT, TG, LDL-c, TC, HbA1c, UA, HT, DM, hsCRP, HOMA-IR, current medications
Kim, B-J (2019) (147)	South Korea	Cross-sectional	Nationally representative surveys (KNHANES 2014-2015), pos-menopausal ♀ and ≥50y ♂ (n=4103)	46.2%	61.7±8.8[a]	HSI (per unit increase)	Dynamo meter	HGS (♂ <28.9, ♀ <16.8)	♂ AOR 1.17 (1.07–1.28), ♀ AOR 1.11 (1.02–1.20)	age, weight, SBP, smoking, resistance exercise, TC, TG, HbA1c, ALT

Oshida, N (2019) (126)	Japan	Cross-sectional	Outpatients followed for lifestyle-related liver diseases (n=253)	46.6%	<30: 21.7%, 31-60: 41.9%, >60: 36.4%	US	Dynamo meter	HGS KES	NAFLD (n=153) vs. non-NAFLD (n=100) HGS: <30y 31.7 vs. 35.6, 31-60y 34.2 vs. 31.4, >60y 29.2 vs. 27.0, NS KES <30y 56.0 vs. 80.5 p<0.01, 31-60y 53.8 vs. 75.2 p<0.01, >60y 51.9 vs. 57.5 NS	NA
Zhang, Y (2019) (123)	China	Cross-sectional	Post-menopausal women in an outpatient clinic, 50–65y (n=96)	NA	59.7±3.6[a]	^{1}H MRS	Dynano meter chair	HGS/weight KES/weight EFS/weight	HGS/weight: r=-0.20, p=0.061 KES/weight: r=-0.24, p=0.022 EFS/weight: NS	HOMA-IR
Hao, L (2020) (135)	China	Cross-sectional	Health survey (Multi-center Application Research on Fitness Test and Exercise Management project of China Health Foundation), 20-60y, male (n=1126)	NA	36.56±8.93	US	Dynamo meter	HGS/weight	OR 0.171 (0.106-0.275), AOR 0.642 (0.503-0.842)	body fat percentage, BMI, SBP, DBP, TC, HDL-c, TG, VO2max >30 mL/kg^{-1}·min^{-1}
Kang, S (2020) (148)	South Korea	Cross-sectional	Nationally representative survey (KNHANES 2014-2016), 20-79y (n=14861)	42.4%	45.6±0.2	HSI (>36.0)	Dynamo meter	HGS/BMI (Q1)	OR 3.62 (3.25-4.03), AOR 1.92 (1.61-2.29)	Age, sex, obesity, DM, HT, dyslipidaemia, HOMA-IR, elevated hs-CRP level
Park, SH (2020) (149)	South Korea	Cross-sectional	Nationally representative surveys (KNHANES 2015), ≥19y (n=3922)	41.9%	♂45.0 (0.5) ♀ 46.9 (0.5)	LFS (>-0.640)	Dynamo meter	HGS/BMI (Q4)	♂ OR 0.19 (0.13-0.27), AOR 0.23 (0.15-0.35); ♀ OR 0.08 (0.05-0.13), AOR 0.20 (0.11, 0.34)	Age, alcohol consumption, smoking, DM, HT, dyslipidemia, CHD, CVD, physical activity, TyG, CRP

[a] Calculated by authors; ♂– male, ♀– female, ^{1}H-MRS – single-voxel proton magnetic resonance spectroscopy, 25[OH]D – 25-hydroxyvitamin D, AGR – android-fat-to-ginoid-fat ratio, ALT – alanine aminotransferase, AOR – adjusted odds ratio, BMI – body mass index (kg/m^2), CHD – coronary heart disease, CVD – cerebrovascular disease, CRP - C-reactive protein, CT - computed tomography, DBP – diastolic blood pressure, DM – diabetes mellitus, DXA – dual-energy x-ray absorptiometry, EFS – elbow flexors strength (kg), HbA1c – glycated hemoglobin, HDL-c – high-density lipoprotein cholesterol, HGS – handgrip strength (kg), HIV – human immunodeficiency viruses, HOMA-IR – homeostasis model of insulin resistance, hsCRP – high sensitivity C-reactive protein, HSI – hepatic steatosis index, HT – hypertension, INSTI – integrase strand transfer inhibitor, IQR – interquartile range, KES – knee extension strength (kg), KNHANES – Korean National Health and Nutrition Examination Survey, LDL-c – low-density lipoprotein cholesterol, LFS – liver fat score, MetS – metabolic syndrome, NA – not applicable, NAFLD – non-alcoholic fatty liver disease, NNRTI – non-nucleoside reverse transcriptase inhibitors, NRTI – nucleoside reverse transcriptase inhibitors, NR – not reported, NS – non-significant, OR – odds ratio, PI – protease inhibitor, Q1 – lowest quartile, Q4 – highest quartile, SBP – systolic blood pressure, SD – standard deviation, TC – total cholesterol, TG – triglycerides, TyG – triglycerides and glucose index, UA – uric acid, US – ultrasound, VO2max – maximal oxygen uptake, y – years

Table 4 – Characteristics and results of studies assessing the association of muscle mass and severity of NAFLD (ordered by variable of assessment of muscle mass, and sample size)

First author (year)	Country	Study type	Setting and population (size)	Male (%)	Age (y), mean±SD or median (IQR)	Assesment of NAFLD severity	Assesment of muscle mass		Association or risk measure	Confounder adjustment
							Method	Parameter		
Tsien, C (2012) (108)	United States	Cross-sectional	Patient with NAFLD on LB (n=74)	NR	NR	LB	CT	TPA	Steatosis (n=19) vs. NASH (n=42) vs. Cirrhosis (n=13): 30.4±9.9 vs. 26.5±85 vs. 22.2±6.4	NA
Issa, D (2014) (109)	United States	Cross-sectional	Patients with NASH on LB (n=50)	NR	NR	LB	CT	TPA at L4	NASH (n=25) vs. Cirrhosis (n=25): 24.8±0.8 vs. 19.3±0.93, p<0.001	NR
Lee, YH (2015) (140)	South Korea	Cross-sectional	Nationally representative survey (KNHANES 2008-2011), ≥20y, NAFLD diagnosed by HSI/CNS/LFS (n=NR)	NR	NR	BARD (≥2), FIB-4 (≥2.67)	DXA	ASM/weight (<32.2% ♂, 25.5% ♀)	Sarcopenic (n=NR) vs. non-sarcopenic (n=NR) BARD: 60% vs. 45%, p <0.001 FIB-4: 22% vs. 14%, p <0.001.	NA
Yamaguchi, A (2015) (152)	Japan	Cross-sectional	Patients with NAFLD (CAP>240) (n=64)	NR	NR	LSM (≥9.0)	BIA	SMM/weight	Lower SMM/weight ♂ p=0.01, ♀ p=0.003	NR
Joo, SK (2016) (110)	South Korea	Cross-sectional	Patients with NAFLD on LB (n=223)	53.4%	52.24±14.87	LB	DXA	ASM/weight	♂ decreased p<0.001, ♀ NS	NR
Kim, W (2016) (101)	South Korea	Cross-sectional	Patients with NAFLD on LB (n=179)	NR	NR	LB (NASH)	BIA	ASM/weight	Q1: ♂ AOR 4.258 (1.273-14.246), ♀ NS	age, MetS, FM, HOMA-IR
Lee, YH (2016) (145)	South Korea	Cross-sectional	Nationally representative survey, ≥20y (KNHANES 2008-2011)	44.9%	55.8±14.3	NFS[c] (Q4), FIB-4 (≥2.67), Forns	DXA	ASM/BMI	NFS: AOR 0.67 (0.49-0.91) FIB-4: AOR 0.73 (0.53-0.99) Forns Index: AOR 0.79 (0.54-1.15)	age, age x ASM/BMI, sex, BMI, WC, HOMA-IR, FPG, TC, TG,

			with NAFLD (LFS≥-0.640) (n=2761)			index[d] (Q4)				AST, ALT, DM, HT, exercise, smoking, eGFR, drinking, residence, history of CVD, CHD, COPD and malignancy
Kallwitz, ER (2017) (143)	United States	Cross-sectional	Nationally representitive survey (NHANES 1999-2014), NAFLD (HSI, FLI, LFS) (n=NR)	NR	NR	NFS	NR	ASM/BMI	Every 1SD decrease: NFS AOR 4.58 (3.04-6.91)	NR
Koo, BK (2017) (102)	South Korea	Cross-sectional	Patients with radiologic evidence of hepatic steatosis and LB, ≥ 18y (Boramae NAFLD registry) (n=240)	48.8%	53.4±14.4[a]	LB, LSM	BIA	ASM/weight (<29% ♂, <22.9% ♀) ASM/BMI (<0.789 ♂, <0.512 ♀)	NASH ASM/weight: OR 2.46 (1.35-4.48), AOR 2.30 (1.08-4.93); ASM/BMI: OR 2.16 (1.13-4.14), AOR 2.33 (1.02-5.34) F≥2 ASM/weight: OR 2.01 (1.12-3.61), AOR 2.05 (1.01-4.16); ASM/BMI: OR 2.86 (1.49-5.35), AOR 2.24 (1.06-4.73)	age, sex, BMI, smoking, HT, DM, TG, HOMA-IR; and TC, HDL-c, ALT and hsCRP for NASH; and platelet and albumin for fibrosis
Osaka, T (2017) (124)	Japan	Cross-sectional	Patients with T2DM, NAFLD on US (n=185)	56.2%	63.9±12.3[a]	LSM	BIA	SMM/weight	β=−0.34, p<0.001 LSM ≥F2 (7.6kPa), per incremental 1% of SMM/weight: AOR 0.66 (0.53-0.80)	age, sex, insulin treatment, HbA1c, AST, ALT, platelet, ferritin, hyaluronic acid and type IV collagen 7S
Peng, TC (2017) (112)	United States	Cross-sectional	Nationally representativ	48.6%	66.71 (mean)	US	BIA	SMM/weight (<37.0% ♂,	SMM/weight: mild steatosis OR 1.33 (1.05-1.69), AOR 1.41 (1.09-	age, sex, race/ethnicity,

			e survey (NHANES 1988-1994), 60-75y, NAFLD on abdominal US (n=2551)					<28% ♀) SMM/height^2 (<10.76 ♂, <6.75 ♀)	1.83); moderate steatosis OR 2.15 (1.71-2.69), AOR 2.22 (1.74-1.83); severe steatosis OR 2.33 (1.73-3.14), AOR 2.30 (1.67-3.17) SMM/height^2: mild steatosis OR 0.74 (0.58-0.93), AOR 0.63 (0.48-0.83); moderate steatosis OR 0.58 (0.47-0.71), AOR 0.52 (0.41-0.67); severe steatosis OR 0.49 (0.37-0.64), AOR 0.44 (0.32-0.61)	TC, 25[OH]D, HbA1c, CRP, UA, physical activity, smoking
Petta, S (2017) (104)	Italy	Cross-sectional	Patients with NAFLD on LB (n=225)	62.7%	48.3±13.4	LB	BIA	ASM/weight (<37% ♂, <28% ♀)	G3 steatosis: AOR 2.02 (1.06-3.83) Ballooning: AOR 1.28 (0.51-3.17) NASH: 0.98 (0.39-2.45) F≥3: AOR 1.76 (1.03-3.73)	G3 steatosis: visceral obesity Ballooning: sex, visceral obesity, FPG>100/DM NASH: sex, age>50, HT, visceral obesity, FPG>100/DM F≥3: age>50, HOMA-IR, HT, NASH, use of Angiotensin-converting enzyme inhibitors/angiotensine II receptor blockers, metformin, calcium channel blockers and statins

Choe, EK (2018b) (137)	South Korea	Cross-sectional	Patients with T2DM and NAFLD on abdominal US (n=2555)	50.9%	56.0±10.4	FIB-4	Physical examination	WCR (T3)	AOR 8.62 (1.39-53.36)	age, sex, BMI, HT, duration of DM, exercise status, smoking and alcohol history, HbA1c, TC, TG, Kitt, and medication history for DM and dyslipidemia
Kapuria, D (2018) (106)	United States	Cross-sectional	Patients with NAFLD on LB (n=60)	58%	45.8±13	LB	CT	TPA/height^2	Advanced steatosis: 619 vs. 454, p=0.006; AOR NR, p=0.009 Fibrosis: NS NASH: NS	Age, sex, HOMA-IR, weight
Kwanten, WJ (2018) (103)	Belgium	Cross-section	Obese patients with NAFLD on LB (n=162)	NR	NR	LB	BIA CT	Muscle mass[b]/weight (<2SD below reference)	NAFL (n=39) vs. <F2 (n=94) vs. ≥F2 (n=29): 23.1% vs. 24.5% vs. 34.5%	NA
Shida, T (2018) (133)	Japan	Cross-sectional	Patients with NAFLD (US and elevated ALT) (n=337)	58.4%	NR	CAP (≥260), LSM (≥12)	BIA	SMM/VFA (Q1)	CAP: OR 1.89 (0.78–4.54), AOR 4.33 (1.35-13.8) LSM: OR 3.64 (0.81–16.4), AOR 7.83 (1.46-41.9)	age, sex and HOMA-IR
Alferink, LJM (2019) (114)	The Netherlands	Cross-sectional	Health survey, european, ≥45y (The Rotterdam Study) with NAFLD on US and data on LSM (n=1126)	47.9%	NR	LSM (≥8.0kPa)	DXA	ASM/height^2	♂ AOR 1.03 (0.65-1.61), ♀ AOR 0.48 (0.25-0.92)	age, study cohorts, weight, height, HOMA-IR, TG, AGR
Chung, GE (2019) (120)	South Korea	Cross-sectional	Routine health	NR	NR	US (severe steatosis)	BIA	ASM/weight	ASM/weight <29 ♂, <22.9 ♀: AOR 1.62 (1.28-2.05)	age, sex, smoking, VFA,

			evaluation, NAFLD on US (n=3699)						ASM/weight Q1: AOR 1.33 (1.25–1.41)	HT, DM, TC, LDL-c, HDL-c, TG
Kang, MK (2019) (111)	South Korea	Cross-sectional	Routine health evaluation, ≥ 20y with NAFLD on abdominal US (n=10711)	52.85%	47.9±11.7	NFS, FIB-4	BIA	ASM/weight (<29% in ♂, <22.9% in ♀) ASM/BMI (<0.789 in ♂, <0.512 in ♀)	NFS ≥-1.455: ASM/weight OR 2.72 (2.29-3.23), AOR 1.64 (1.34-1.99); ASM/BMI OR 3.00 (2.48-3.61), AOR 2.01 (1.63-2.46) NFS≥0.676: ASM/weight OR 3.98 (1.95-7.44), AOR 2.68 (1.28-5.59); ASM/BMI OR 4.46 (2.12-8.51), AOR 3.12 (1.51-6.46) FIB-4>1.30: ASM/weight OR 1.52 (1.25-1.84), AOR 1.26 (1.03-1.54); ASM/BMI OR 2.39 (1.96-2.90), AOR 2.00 (1.63-2.45) FIB-4>2.67: ASM/weight OR 2.04 (1.14-3.40), AOR 1.58 (0.87-2.85); ASM/BMI OR 2.20 (1.17-3.77), AOR 1.62 (0.86-2.98)	sex, HT, obesity, TC, TG, HDL-c, hsCRP; and FPG for NFS; and DM, albumin, GGT for FIB-4
Mizuno, N (2019) (107)	Japan	Longitudinal	Patients with histological diagnosis of NAFLD on LB (n=219; 12 months follow-up 139)	46.7%	58 (17-84)	LB ΔALT (decrease)	BIA	SMM/height^2 SMM/FM	At baseline Simple steatosis vs. NASH SMM/height^2: 7.29 (4.89-10.07) vs. 7.29 (4.86-10.43), p=0.689; SMM/FM 0.88 (0.25-3.76) vs. 0.72 (0.38-1.70), p=0.015 Fibrosis stage NS At follow-up ΔALT: SMM/FM ♂ AHR 10.99 (1.437-83.33), ♀ AHR 6.849 (1.443-32.26)	At baseline: NA At follow-up: age, HT, hyperlipidemia, DM, GGT, platelet count, fibrosis stage, NAS
Seko, Y (2019) (105)	Japan	Longitudinal	Patients with NAFLD on LB (n= 156 at baseline; n=121 at 12 months follow-up)	47.4% (43.0% follow-up)	57.5 (17–84) [56 (17–79) for follow-up]	LB ΔALT (decrease >30%)	BIA	ASM/BMI ASM/FM ASM/height^2	At baseline F<2: ASM/height^2 p=0.157, ASM/BMI p=0.008 ASM/FM p=0.047; NAS<6: ASM/height^2 p=0.097, ASM/BMI p=0.019 ASM/FM p=0.035 At follow-up, ΔALT:	At baseline: none At follow-up: age, sex, platelet count, fibrosis, NAS, ΔASM/BMI, ΔASM/FM

									ΔASM/BMI AOR 1.354 (0.362-5.066); ΔASM/FM AOR 7.406 (1.796–30.54)	
Seo, DH (2019) (117)	South Korea	Cross-sectional	Patients with MetS, 30-64y (Seoul Metabolic Syndrome Cohort), NAFLD on US (n=1278)	51.3%	55.8±11.0[a]	US	BIA	ASM/weight (<29% ♂, <22.9% ♀) ASM/BMI (<0.789 ♂, <0.512 ♀)	Sarcopenic vs. non-sarcopenic Moderate-to-severe NAFLD: ♂ (n=676) 87.8%[a] vs. (n=1484) 72.4%[a], ♀ (n=564) 77.3%[a] vs. (n=1486) 69.2%[a]	NA
Shida, T (2019) (134)	Japan	Longitudinal	Patients with NAFLD on abdominal US (n=92)	39.1%	55.5±14.3	ΔLSM, ΔCAP	BIA	ΔSMM/VFA	Worsened (n=32) vs. stable (n=46) vs. improved (n=14) ΔSMM/VFA ΔLSM: 1.3 vs. 0.6 vs. 0.9, NS ΔCAP: 27.9 vs. 1.0 vs. -20, p<0.01	NA
Wijarnpreecha, K (2019) (122)	United States	Cross-sectional	Nationally representative surveys (NHANES 1988-1994), 20-74y, NAFLD on US (n=4188)	47.1%	45.4±0.43	NFS	BIA	SMM/weight (<37% in ♂, <28% in ♀)	NFS>0.676: OR 5.20 (3.20-8.44), AOR 1.79 (1.18-2.72) NFS>0.12 in patients aged ≥65 years: OR 4.57 (3.19-6.54), AOR 1.74 (1.22-2.48)	age, sex, ethnicity, WC, DM, smoking, HT, TC, sedentary physical activity, 25[OH]D deficiency
Hyun Kim, K (2020) (128)	South Korea	Cross-sectional	Patients with NAFLD on US (n=957)	60.1%	51.4±14.2	CAP, LSM	BIA	ASM/BMI (<0.789 ♂, <0.521 ♀)	Sarcopenic (n=123) vs. non-sarcopenic (n=834): CAP 309.5±39.6 vs. 307.9±40.2, p=0.680; LSM 8.4±6.0 vs. 6.6±3.5, p=0.001	NA

[a] Calculated by authors, [b] not specified if ASM or SMM, [c] serum albumin was not used due to lack of data, [d] only 1969 subjects were analyzed due to missing GGT values; ♂– male, ♀– female, 25[OH]D – 25-hydroxyvitamin D, AGR – android-fat-to-ginoid-fat ratio, AHR – adjusted hazard ratio, ALT – alanine aminotransferase, AOR – adjusted odds ratio, ASM – appendicular skeletal muscle mass (kg), AST – aspartate aminotransferase, BARD – BARD score, BIA – bioelectrical impedance analysis, BMI – body mass index (kg/m^2), CAP – controlled attenuation parameter, CHD – coronary heart disease, CNS – comprehensive NAFLD score, COPD – chronic obstructive pulmonary disease, CRP - C-reactive protein, CT - computed tomography, CVD – cerebrovascular disease, DM – diabetes mellitus, DXA – dual-energy X-ray absorptiometry, eGFR – estimated glomerular filtration rate, F – fibrosis grade, FIB-4 – fibrosis-4 index, FM – fat mass (kg), FLI – Fatty Liver Index, FPG – fasting plasma glucose, G3 – grade 3, GGT – gamma-glutamyltransferase, HbA1c – glycated hemoglobin, HDL-c – high-density lipoprotein cholesterol, HOMA-IR – homeostasis model of insulin resistance, hsCRP – high sensitivity C-reactive protein, HSI – hepatic steatosis index, HT – hypertension, IQR – interquartile range, KNHANES – Korean National Health and Nutrition Examination Survey, L4 – 4th lumbar vertebrae, LB – liver biopsy, LDL-c – low-density lipoprotein cholesterol, LFS – liver fat score, LSM – liver stiffness, MetS – metabolic syndrome, NA – not applicable, NAFL – non-alcoholic fatty liver, NAFLD – non-alcoholic fatty liver disease, NAS – NAFLD activity score, NASH – non-alcoholic steatohepatitis, NFS – NAFLD fibrosis score, NHANES – National Health and Nutrition Examination Survey, NR – not reported, NS – non-significant, OR – odds ratio, Q1 – lowest quartile, Q4 – highest quartile, SD – standard deviation, SMM – skeletal muscle mass, T2DM – type 2 diabetes mellitus, T3 – highest tercile, TC – total cholesterol, TG – triglycerides, TPA – total psoas muscle area (cm^2), UA – uric acid, US – ultrasound, VFA – visceral fat area (cm^2), WC – waist circumference, WCR – waist-to-calf ratio, y – years

Table 5 – Characteristics and results of studies assessing the association of muscle strength and/or performance and severity of NAFLD

First author (year)	Country	Study type	Setting and population (size)	Male (%)	Age (y), mean±SD or median (IQR)	Assesment of NAFLD severity	Assesment of muscle strength or performance		Association or risk measure	Confounder adjustment
							Method	Parameter		
Peng, TC (2017) (112)	United States	Cross-sectional	Nationally representative survey (NHANES 1988-1994), 60-75y (n=2551)	48.6%	66.71 (mean)	US	Physical examination	Gait speed	Mild steatosis OR 1.29 (1.01-1.65), AOR 1.12 (0.86-1.45); moderate steatosis OR 1.32 (1.07-1.64), AOR 1.17 (0.92-1.47); severe OR 1.15 (0.88-1.50), AOR 0.94 (0.70-1.25)	age, sex, race/ethnicity, TC, 25[OH]D, HbA1c, CRP, UA, physical activity, smoking
Cruz, JF (2019) (136)	Brazil	Cross-sectional	Patients with NAFLD on US (n=59)	NR	NR	US	Dynamometer	EFS/BMI KES/BMI	Grade 1 vs. Grade 2 vs. Grade 3 EFS/BMI: ♂ 2.70±0.55 vs. 2.59±0.62 vs. 2.12±0.25, ♀ 3.12±0.80 vs. 2.20±0.60 vs. 1.67±0.46; p=0.028 KES/BMI: ♂ 3.93±1.05 vs. 4.01±0.91 vs. 2.29±0.63, ♀ 3.06±1.44 vs. 2.90±1.10 vs. 2.30±0.77; p=0.013	NA
Kang, S (2020) (148)	South Korea	Cross-sectional	Nationally representative survey (KNHANES 2014-2016), 35-65y, HSI>36 (n=2029)	42.4%	45.6±0.2	FIB-4 ≥1.30, BARD ≥2	Dynamometer	HGS/BMI (Q1)	FIB-4: OR 1.66 (1.01-2.49), AOR 1.35 (0.75-2.45) BARD: OR 1.81 (1.30-2.51), AOR 1.68 (1.07-2.62)	Age, sex, obesity, DM, HT, dyslipidaemia, HOMA-IR, elevated hs-CRP level
Park, SH (2020) (149)	South Korea	Cross-sectional	Nationally representative surveys (KNHANES 2015), ≥19y, NAFLD (LFS >-0.640) (n=946)	NR	♂45.0 (0.5) ♀ 46.9 (0.5)	FIB-4, NFS	Dynamometer	HGS/BMI	FIB-4: Q1 1.38 vs. Q4 0.92, p<0.05 NFS: Q1 vs. Q4 p<0.001	NA

25[OH]D – 25-hydroxyvitamin D, AOR – adjusted odds ratio, BMI – body mass index (kg/m^2), CRP - C-reactive protein, EFS – elbow flexors strength (kg), FIB-4 – Fibrosis-4 Index for Liver Fibrosis, HbA1c – glycated hemoglobin, HGS – handgrip strength (kg), HSI – hepatic steatosis index, IQR – interquartile range, IR – insulin resistance, KES – knee extension strength (kg), KNHANES – Korean National Health and Nutrition Examination Survey, LFS – liver fat score, NA – not applicable, NAFLD – non-alcoholic fatty liver disease, NFS – NAFLD fibrosis score, NHANES – National Health and Nutrition Examination Survey, NR – not reported, OR – odds ratio, Q1 – lowest quartile, Q4 – highest quartile, SD – standard deviation, TC – total cholesterol, UA – uric acid, US – ultrasound, y – years

Table 6 – Characteristics and results of studies assessing the association of sarcopenia and presence or severity of NAFLD

First author (year)	Country	Study type	Setting and population (size)	Male (%)	Age (y), mean ± SD or median (IQR)	Diagnosis of NAFLD	Definition of sarcopenia	Association or risk measure	Confounder adjustment
Peng, TC (2017) (112)	United States	Cross-sectional	Nationally representative survey (NHANES 1988-1994), 60-75y (n=2551)	48.6%	66.71 (mean)	US	SMM/weight (BIA) <37.0 ♂, <28 ♀ or SMM/height^2 (BIA) <10.76 in ♂, <6.75 in ♀ or SMM/BMI (BIA) <0.99 ♂, <0.58 ♀ or SMM (BIA) <26.51 ♂, <16.14 ♀ and Gait speed ≤0.08	SMM/weight: mild steatosis OR 1.44 (1.13-1.83), AOR 1.43 (1.11-1.86); modereate steatosis OR 1.94 (1.57-2.39), AOR 1.88 (1.50-2.37); severe steatosis OR 1.67 (1.27-2.18), AOR 1.52 (1.14-2.04) SMM/height^2: mild steatosis OR 1.05 (0.82-1.36), AOR 0.92 (0.70-1.21); moderate steatosis OR 0.79 (0.62-0.99), AOR 0.72 (0.56-0.92); severe steatosis OR 0.68 (0.50-0.93), AOR 0.64 (0.46-0.90) SMM/BMI: mild steatosis AOR 1.67(1.22-2.29), moderate steatosis AOR 1.99 (1.51-2.62), severe steatosis AOR 1.77 (1.25-2.50) SMM: mild steatosis AOR 1.00 (0.72-1.38), moderate steatosis AOR 0.64 (0.46-0.89), severe steatosis AOR 0.78(0.52-1.18)	age, sex, race/ethnicity, TC, 25[OH]D, HbA1c, CRP, UA, physical activity, smoking
Zhai, Y (2018) (131)	China	Cross-sectional	Inpatients, >60y (n=494)	43.7%	71.28 (mean)	US	ASM/height^2 (DXA) <7.0 ♂, <5.4 ♀ HGS <26 ♂, <18 ♀ gait speed <0.8	*R*=-0.15, *p*=0.001	age, sex, BMI, HT, DM, HbA1c, high UA hematic disease, hs-CRP, ALT, AST, TC, TG, LDL-c, HDL-c
Alferink, LJM (2019) (114)	The Netherlands	Cross-sectional	Health survey, european, ≥45y	43.0%	69.3±9.2	US	ASM/height^2 (DXA) ≤7.25 ♂, ≤5.67 ♀ and	Normal weight: ♂ AOR 2.20 (0.94-5.13), ♀ AOR 1.23 (0.49-3.07)	age, study cohorts, weight, height, HOMA-IR, TG, AGR

			(The Rotterdam Study) (n=4609)				HGS ♂ ≤29 kg for BMI ≤24 kg/m^2, ≤30 kg for BMI 24.1-28 kg/m^2, ≤32 kg for BMI >28 kg/m^2; ♀ ≤17 kg for BMI ≤23 kg/m^2, ≤17.3 kg for BMI 23.1-26 kg/m^2, ≤18 kg for BMI 26.1-29 kg/m^2, ≤21 kg for BMI >29 kg/m^2 or Gait speed ♂ <0.65 if height ≤173 cm, <0.76 if height >173 cm; ♀ <0.65 if height ≤159 cm, <0.76 if height >159 cm	Overweight: ♂ AOR 1.88 (0.95-3.72), ♀ AOR 0.57 (0.14-2.41)	

25[OH]D – 25-hydroxyvitamin D, AGR – android-fat-to-ginoid-fat ratio, ALT – alanine aminotransferase, AOR – adjusted odds ratio, ASM – appendicular skeletal muscle mass (kg), AST – aspartate aminotransferase, BIA – bioelectrical impedance analysis, BMI – body mass index (kg/m^2), CRP – C-reactive protein, DM – diabetes mellitus, DXA – dual-energy X-ray absorptiometry, HbA1c – glycated hemoglobin, HDL-c – high-density lipoprotein cholesterol, HGS – handgrip strength, hsCRP – high sensitivity C-reactive protein, HOMA-IR – homeostasis model of insulin resistance, HT – hypertension, IQR – interquartile range, LDL-c – low-density lipoprotein cholesterol, NAFLD – non-alcoholic fatty liver disease, NHANES – National Health and Nutrition Examination Survey, OR – odds ratio, SD – standard deviation, SMM – skeletal muscle mass, TC – total cholesterol, TG – triglycerides, UA – uric acid, US – ultrasound, y – years

Table 7- Modified Newcastle-Ottawa Scale for assesment of bias

Longitudinal studies								
First author (year)	Selection				Comparability	Outcome		
	Representativeness of the exposed cohort[a]	Selection of the non exposed cohort[b]	Ascertainment of exposure[c]	Demonstration that outcome of interest was not present at start of study[d]	Comparability of cohorts[e]	Assessment of outcome[f]	Was follow-up long enough for outcomes to occur[g]	Adequacy of follow up of cohorts[h]
Rachakonda, V (2017) (113)	*	*	*	*	0	*	*	0
Kim, G (2018) (141)	*	*	*	*	**	*	*	*
Lee, MJ (2019) (116)	*	*	*	*	**	*	*	*
Mizuno, N (2019) (107)	*	*	*	*	**	*	*	*
Seko, Y (2019) (105)	*	*	*	*	**	*	*	*
Shida, T (2019) (134)	*	*	*	*	0	*	*	*
Cross-sectional studies								
Authors	Selection				Comparability	Outcome		
	Representativeness of the sample[i]	Sample size[j]	Non-respondents[k]	Ascertainment of the exposure[l]	Comparability of subjects in different outcome groups[m]	Assessment of outcome[n]	Statistical test[o]	
Tsien, C (2012) (108)	0	0	0	**	0	*	0	
Choi, YJ (2013) (118)	*	*	0	**	**	*	*	
Moon, JS (2013) (139)	*	*	0	**	**	*	*	
Hong, HC (2014) (119)	*	*	0	**	**	*	*	
Issa, D (2014) (109)	0	0	0	**	0	*	0	
Lee, YH (2015) (140)	*	*/0[p]	0	**	**/0[p]	*	*	
Yamaguchi, A (2015) (152)	0	0	0	**	0	*	0	
Hashimoto, Y (2015) (151)	*	*	0	**	**	*	*	
Joo, SK (2016) (110)	*	0	0	**	0	*	0	

Kim, HY (2016) (138)	*	*	0	**	**	*	*
Kim, W (2016) (101)	*	*	0	**	**/0 [p]	*	*/0 [p]
Lee, YH (2016) (145)	*	*	0	**	**	*	*
Poggiogalle E, (2016) (150)	*	*	0	*	**	*	*
Shen, H (2016) (130)	*	0	0	**	0	*	*
Kallwitz, ER (2017) (143)	*	0	0	**	0	*	0
Koo, BK (2017) (102)	*	0	0	**	**	*	*
Osaka, T (2017) (124)	*	0	0	**	**	*	*
Peng, TC (2017) (112)	*	*	0	**	**	*	*
Petta, S (2017) (104)	*	*	0	**	**/* [p]	*	*
Choe, EK (2018a) (125)	*	*	0	**	**	*	*
Choe, EK (2018b) (137)	*	*	0	*	**	*	*
Kapuria, D (2018) (106)	*	0	0	**	**	*	0
Kwanten, WJ (2018) (103)	*	*	0	**	0	*	*
Lee, K (2018) (146)	*	*	0	**	**	*	*
Shida, T (2018) (133)	*	*	0	*	**	*	*
Yerragorla, P (2018) (100)	*	0	0	**	0	*	0
Zhai, Y (2018) (131)	*	*	0	**	**	*	*
Alferink, LJM (2019) (114)	*	*	0	**	**	*	*/0 [p]
Chen, VL (2019) (115)	*	*	0	**	**/0 [p]	*	*/0 [p]
Chung, GE (2019) (120)	*	*	0	**	**	*	*
Cruz, JF (2019) (136)	*	*	0	**	**/0 [p]	*	0
Debroy, P (2019) (129)	*	0	0	**	**/0 [p]	*	*/0 [p]
Gan, D (2019) (121)	*	*	0	**	**	*	*
Gerber, L (2019) (144)	*	*	0	**	**	*	0
Hsing, JC (2019) (142)	*	*	0	**	**	*	*
Kang, MK (2019) (111)	*	*	0	**	**	*	*
Kim, B-J (2019) (147)	*	*	0	**	**	*	*
Oshida, N (2019) (126)	*	0	0	**	0	*	0
Seo, DH (2019) (117)	*	*	0	**	**/0 [p]	*	*

Su, X (2019) (132)	*	*	0	**	**	*	*
Wijarnpreecha, K (2019) (122)	*	*	0	**	**	*	*
Zhang, Y (2019) (123)	*	*	0	**	*	*	*
Hao, L (2020) (135)	*	*	0	**	*	*	*
Hyun Kim, K (2020) (128)	*	*	0	**	0	*	0
Kang, S (2020) (148)	*	0	0	**	**	*	*
Park, SH (2020) (149)	*	*	0	**	**/0 [p]	*	*/0 [p]
Tanaka, M (2020) (127)	*	*	0	**	**	*	*

Point (*) if: a- truly or somewhat representative, b- drawn from the same population as the exposed cohort, c- validated method, d- yes, e- study controls for age (another point for any additional factor), f- validated method, g- follow-up was ≥12 months, h- lost to follow-up <20% or description provided of those lost, i- truly or somewhat representative of target population, j- justified and satisfactory or adequately powered to detect a difference (>10 events per variable in multivariable analysis, k- response rate ≥60% and comparability between respondents and non-respondents characteristics is established, l- adequate method (another point if validated/recommended), m- controls for age, another point for other factors, n- validated method, o- clearly described and appropriate, either odds ratio with 95% interval confidence or *p*-value, or correlation coefficient and *p*-value, p- depending on outcome/exposure analysed

5.4. Discussion

In this systematic review, we intended to describe the association between muscle mass, strength, and performance, and the presence and severity of NAFLD. Most studies found an association between low muscle mass and the presence and/or severity of NAFLD when these measures were adjusted for weight or BMI but not for height. There is ongoing debate as to which adjustment is optimal in differing situations.(153) This is of particular importance since these tools were initially designed to assess sarcopenia in the elderly and frail. In the presence of overweight or obesity, the use of weight or BMI indexing is likely more informative of body composition, while also allowing for the inclusion of patients in a larger range of body sizes. Moreover, BMI indexing allows for the inclusion of patients with "sarcopenic obesity", which may be more relevant in NAFLD, a condition in which most patients have excess weight, and, as such, are more difficult to diagnose sarcopenia.(154) Height indexing can mask sarcopenia in overweight individuals and may underplay the interaction of muscle and fat tissues.

Overall, there was an association of low muscle strength and NAFLD, regardless of method of strength measurement. To the best of our knowledge, our review is the first to analyze this association. In most recent European Working Group on Sarcopenia in Older People (EWGSOP) guidelines, strength has been considered the defining feature of sarcopenia.(7) This has come from evidence that low strength is the muscle parameter most often associated with adverse patient outcomes and, as such, the most useful in clinical practice.(7).

Most studies adjusted for insulin resistance, either as DM, levels of fasting glucose, glycated hemoglobin, homeostasis model of insulin resistance, insulin sensitivity index, or short insulin tolerance test. Sarcopenia has long been associated both with T2DM and the MetS.(155, 156). Myosteatosis, the infiltration of lipids in skeletal muscle tissue, has been associated with IR and with lower muscle strength; as such, IR may be one of the unifying factors of NAFLD and sarcopenia. (157)

Vitamin D was only considered in four studies. Levels are lower and deficiency is more common in patients with NAFLD (158). Vitamin D is also intimately connected to

sarcopenia, with receptors being expressed in skeletal muscle cells and mediating genomic and non-genomic effects that translate into reduced muscle performance with low levels of this hormone.(159)

Analysis by sex was only performed in a minority of studies; however, most other utilized sex-specific cut-offs for low muscle mass or strength, which limited this bias. Men have a higher percentage of lean muscle mass than women. The age-related decrease of sex hormones, particularly of testosterone, contributes to the loss of muscle mass and function.(160) The prevalence of NAFLD is higher in men than women, and, in women, is higher after menopause.(161)

Only 15 of the 53 studies were from non-Asian populations; this is important as extrapolating these results might be biased by several factors. NAFLD is a more recent phenomenon in Asia than in Europe and North America.(162). Body fat distribution is different in Asians: abdominal deposition of fat is more common and total body fat is several percentage points higher for the same BMI compared with other ethnicities.(163) As the etiology of NAFLD is still not completely understood, other genetic and cultural factors might also be at play. Nevertheless, studies in western populations have also shown the same tendency of associations.

Seven studies used the Korea National Health and Nutrition Examination Survey (KNHANES), varying the years of inclusion and variables used, but with data overlap; the same is valid for the United States equivalent, the National Health and Nutrition Examination Survey (NHANES). This may have created a bias given the similar methodology and duplication of data used. In addition, some of these data were collected over 30 years ago and may not reflect current trends of lifestyle habits, obesity, and body composition.

An important limitation of this review was the high heterogeneity of definition of variables of interest, providing only two or less studies for each assessment method and outcome definition with extractable information, and precluding a meta-analysis. This was particularly noticeable in methods of assessment of muscle mass. The methods used were almost always in accordance with recommendations but were widely diverse. While the authors understand that methodology can be limited by local availability,

further standardization would allow better comparability between studies and a more robust body of evidence. While language restrictions were established in the selection of studies, none were excluded for this reason only.

Our review is not the first to address the relationship between muscle mass and NAFLD. However, we were alone in including unpublished poster abstracts, which composed 21% of studies included. The exclusion of such a significant number of studies may pose a selection bias. Overall, studies that represented unpublished posters tended to be smaller and to use biopsy for NAFLD diagnosis and staging, as opposed to the studies more frequently published which were large cohort studies that relied on equations or other non-invasive methods.

Increasingly, the definition MAFLD is being used instead of NAFLD, both in clinical and academic settings. This represents a change in population that has not been included in these studies. The addition of other conditions leading to fatty liver disease may obscure changes associated with obesity, diabetes, and other metabolic abnormalities.

5.5. Conclusion

In conclusion, there is a significant number of studies that point to an association of low muscle mass and low muscle strength with the presence and severity of NAFLD. However, the high heterogeneity of methods of assessment of these variables is a hindrance for the progression of this field. Furthermore, the pathophysiological bases and consequences of these associations need to be examined to determine their implication in clinical practice. The authors suggest that specific guidance be issued regarding sarcopenia in NAFLD. Considerations should also be made if MAFLD becomes the prevalent classification, as some have suggested.

6. Associations between muscle mass, strength, and performance and non-alcoholic fatty liver disease

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ORIGINAL ARTICLE

Associations between muscle mass, strength, and performance and non-alcoholic fatty liver disease

Joana RIGOR [1, 2] *, Raquel VASCONCELOS [3], Rogério LOPES [3], Teresa MOREIRA [3], Pedro BARATA [4, 5, 6], Daniela MARTINS-MENDES [2, 6, 7, 8]

[1]Department of Internal Medicine, Vila Nova de Gaia/Espinho Hospital Center, Vila Nova de Gaia, Portugal; [2]Department of Biomedicine, Faculty of Medicine of the University of Porto, Porto, Portugal; [3]Department of Radiology, Vila Nova de Gaia/Espinho Hospital Center, Vila Nova de Gaia, Portugal; [4]Faculty of Health Sciences, Fernando Pessoa University, Porto, Portugal; [5]Department of Pathology, Porto University Hospital, Porto, Portugal; [6]I3S – Institute Health Research and Innovation, University of Porto, Porto, Portugal; [7]Department of Internal Medicine, Fernando Pessoa University Hospital, Porto, Portugal; [8]LaBMI - Biotech solutions, PORTIC - Porto Research, Technology and Innovation Center, Polytechnic Institute of Porto, Porto, Portugal

*Corresponding author: Joana Rigor, Service of Internal Medicine, Vila Nova de Gaia/Espinho Hospital Center, Rua Conceição Fernandes s/n, 4434-502 Vila Nova de Gaia, Portugal. E-mail: rigorj.md@gmail.com

ABSTRACT

BACKGROUND: Non-alcoholic fatty liver disease (NAFLD) is a rising global health issue. The influence of muscle in its pathophysiology has recently gained attention. Our aim was to investigate the association of low muscle mass, strength, and performance with the presence and severity of NAFLD.

METHODS: Patients with metabolic syndrome followed in an outpatient clinic, were consecutively included, between April 1st and December 31st, 2019. Abdominal ultrasound for the diagnosis of NAFLD, NAFLD fibrosis score (NFS) and Fibrosis-4 Index (FIB-4) for determination of significant fibrosis, dual-energy X-ray absorptiometry for calculation of skeletal muscle index (SMI = appendicular skeletal mass / weight x100) and sarcopenic index (SI = appendicular skeletal mass / Body Mass Index), and the Short Physical Performance Battery for muscle strength and performance assessment were performed. Sarcopenia was defined as low muscle strength and low SMI or SI.

RESULTS: A total of 157 patients were included, of which 68.8% had NAFLD, 66.2% low SMI, 50.3% low SI, 16.6% low performance and 11.5% low strength. In patients with NAFLD, prevalence of significant fibrosis by NFS was 15.7%. Low SMI was associated with presence of NAFLD when adjusted for age, sex, type 2 diabetes mellitus, hypertension, and dyslipidemia, but not for body mass index and waist circumference. Low SMI, low SI, and sarcopenia were associated with significant fibrosis in univariate analysis; the small number of events precluded a multivariable analysis.

CONCLUSIONS: Low SMI was associated with NAFLD independently of demographics and comorbidities but not of other parameters of body composition. This contrasts with most studies published on this matter.

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KEY WORDS: Non-alcoholic fatty liver disease; Fibrosis; Sarcopenia; Muscle strength; Body composition.

Non-alcoholic fatty liver disease (NAFLD) is on the rise and is poised to be the predominant liver disease in the western world, with a prevalence of around 25% in the general population and >50% in patients with type 2 diabetes mellitus (T2DM), obesity, and other features of the metabolic syndrome.[1] Furthermore, NAFLD represents up to a third of patients referred to hepatologists for increased transaminases without viral hepatitis.[2] NAFLD presents as a spectrum, from simple steatosis to cirrhosis, with unfavorable outcomes related to fibrosis stage.[1] The gold standard for staging is liver biopsy; however, given the high prevalence of NAFLD,

the benign course in its early stages, and the risks associated with liver biopsy, several non-invasive tools have been recommended to identify patients with advanced liver fibrosis.[3] Like with other causes of liver disease, transient elastography can provide an estimation of fibrosis stage in NAFLD.[4] However, this method is subject to local availability, and out of reach of most primary care settings. Non-invasive scoring systems, that use readily available demographic and clinical variables have been developed. Of these, the NAFLD fibrosis score (NFS)[5] and the Fibrosis-4 Index for Liver Fibrosis (FIB-4)[6-8] are most commonly recommended.

Despite NAFLD being closely related to obesity, there are some patients with obesity that do not present with NAFLD or other metabolic disorders.[9] Furthermore, some patients with NAFLD have normal body mass index (BMI).[10] As such, there has been increasing interest in studying possible factors associated with these discrepancies, to better determine populations at risk and to point to possible pathways for pharmacological treatment.[11, 12]

Exercise has been associated with protection and recovery from metabolic syndrome, in ways not dependent on weight loss, indicating a metabolic role of skeletal muscle beyond energy expenditure.[13] In fact, the skeletal muscle is an endocrine organ, able to produce specific cytokines, called myokines, that modulate the interaction with other organs, namely the liver, the pancreas and the adipose tissue.[14, 15] The skeletal muscle also plays a pivotal role in glucose uptake and insulin resistance, in parallel to the liver. Accordingly, low muscle mass has been associated with impairment in insulin sensitivity and glucose homeostasis.[16]

Low muscle mass is common in patients with cirrhosis, and it is associated with negative outcomes in this population.[17, 18] Interestingly, patients with NAFLD cirrhosis have been found to have a higher prevalence of low muscle mass than patients with viral or alcohol related cirrhosis.[19]

Several papers have been published analyzing the relationship of "sarcopenia" and the presence and severity of NAFLD.[20, 21] However, incorrect terminology has plagued this field, with the term sarcopenia being used as a misnomer for low muscle mass. Indeed, sarcopenia represents muscle dysfunction, translating clinically as low muscle strength, which is accompanied by low muscle quantity or quality. The severity of sarcopenia is given by muscle performance.[22]

Our studied aimed to examine the relationship between sarcopenia and its individual parameters (low muscle mass, low muscle strength, and low muscle performance) with the presence and severity of NAFLD.

Materials and methods

A cohort study was performed, with consecutive inclusion of patients with metabolic syndrome, aged 18 to 75 years-old, followed at an Internal Medicine outpatient clinic, in Vila Nova de Gaia, Portugal, between Abril 1st and December 31st, 2019. Metabolic syndrome was defined as the presence of at least three of the following five criteria: waist circumference ≥88 cm for women and ≥102 cm for men, triglycerides ≥150 mg/dL or treatment for elevated triglycerides, high-density lipoprotein (HDL) <40 mg/dL for men or <50 mg/dL for women or treatment for low HDL, blood pressure ≥130/85 mmHg, history of hypertension or treatment for hypertension, fasting plasma glucose ≥110 mg/dL or history of T2DM or treatment for T2DM. Exclusion criteria included significant alcohol use (>20 g in women and >30 g in men), cirrhosis (as defined by clinical or imagological signs), known liver disease of other etiology, infection by hepatitis B, hepatitis C or human immunodeficiency virus, type 1 diabetes mellitus, active cancer, autoimmune disorders, chronic kidney disease stage ≥3b, and major amputation. The study was authorized by the hospital's Ethics Committee and patients signed an informed consent on inclusion.

Data on medical history and current medication and supplement use were collected. T2DM was defined as history of T2DM, treatment for T2DM, or glycated hemoglobin ≥6.5%. Hypertension was defined as history of hypertension, treatment for hypertension, or blood pressure ≥130/85 mmHg. Dyslipidemia was defined as HDL <40 mg/dL for men or <50 mg/dL for women, triglycerides ≥150 mg/dL, or treatment for dyslipidemia. Physical examination included

blood pressure, heart rate, weight (in kilograms), height (in meters), and waist circumference (in centimeters). BMI was calculated by dividing weight by squared height. To determine physical performance, the Short Physical Performance Battery (SPPB), a composite test of gait speed, balance and strength, was applied; chair stand test was part of the SPPB and was also analyzed alone to assess lower body strength, as per recommendations.[22] The presence of NAFLD was determined by abdominal ultrasound by a skilled radiologist. FIB-4 and NFS were calculated for each patient with NAFLD, with cut-offs for significant fibrosis of >2.67 and >0.675, respectively. Dual-energy x-ray absorptiometry (DXA) was used for body composition analysis, which included appendicular skeletal mass (ASM) and fat percentage.[23] Skeletal mass index (SMI), in percentage, was calculated by dividing ASM by weight and multiplying by 100.[22] Sarcopenic Index (SI) was also obtained, dividing ASM by Body Mass Index (BMI). Low SMI was defined as <22.9% for women and <29.0% for men, and low SI as <0.512 for women and <0.789 for men.[24] An SPPB of ≤8 was indicative of poor performance and a chair stand test >15 seconds indicative of low strength.[22] Sarcopenia was determined by low strength and low SMI or SI.[22]

Statistical analysis

In descriptive analysis, continuous variables with normal distribution were expressed as mean and standard deviation, continuous variables with non-normal distribution as median and interquartile range (IQR), and categorical variables as absolute number and frequencies. For univariate analysis, Student's *t*-Test for independent samples or Mann-Whitney Test were used, according to the variable distribution. In multivariate analysis, adjusted odds ratio (AOR) with the respective 95% confidence intervals (CI) were calculated using logistic regression and an Enter selection approach. Three different models were used: model 1 included age and gender; model 2 included the variables in model 1 and T2DM, hypertension, and dyslipidemia; model 3 included the variables in model 2 and BMI and waist circumference. A p value of less than .05 was considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics for Windows v. 27.0 (IBM Corp., Armonk, NY, USA).

Results

A total of 157 patients were included, all Caucasian, of which 68.8% had NAFLD, 66.2% low SMI, 50.3% low SI, 16.6% low performance and 11.5% low strength. The population characteristics are described in Table I. Patients with NAFLD had higher BMI and waist circumference but similar total fat percentage when compared to patients without NAFLD. They were also more likely to have T2DM, hypertension, and dyslipidemia. Low SMI was present in 75.0% of patients with NAFLD, as opposed to 46.9% of those without (P<0.001). There was no statistically significant difference between the groups regarding low SI (P=0.231), low muscle strength (P=0.061) or low muscle performance (P=0.365).

In multivariable analysis (Table II), low SMI remained associated with NAFLD when adjusting for demographic variables (age and sex) and comorbidities (T2DM, hypertension, and dyslipidemia), but not other body composition data (BMI and waist circumference). Sarcopenia, as defined with SMI, did not maintain its association with NAFLD on multivariable analysis (Table III).

In patients with NAFLD (N.=108), only one (0.9%) had significant fibrosis according to FIB-4. As such, only NFS was used for the analysis, with a prevalence of significant fibrosis of 15.7%. Table IV describes the characteristics of patients with NAFLD, with and without significant fibrosis by NFS. Patients with significant fibrosis were older, had a higher BMI and a higher percentage of T2DM. Regarding muscle mass, the prevalence of low SMI and low SI was 100% and 88.2%, respectively, in patients with significant fibrosis, versus 70.3% and 47.3% in those without, a statistically significant difference. There was also a higher percentage of sarcopenia, using either SMI or SI, in patients with significant fibrosis. Given the small number of events, a multivariable analysis was not performed.

TABLE I.—*Characteristics of the population divided by presence of NAFLD.*

Characteristics	All (N.=157)	With NAFLD (N.=108)	Without NAFLD (N.=49)
Sex, female	81 (51.6%)	50 (46.3%)	31 (63.3%)
Age	67 (59-71)	67 (59-71)	66 (56-71)
BMI	31.2 (27.7-35.8)	32.5 (29.2-36.0)***	28.1 (26.1-31.9)***
Waist circumference	106.5 (98-116)	110.3±11.4***	98.5±11.2***
Total fat percentage	39.8 (34.3-46.6)	39.7 (34.8-47.0)	40.4 (32.3-44.6)
Obesity	89 (56.7%)	73 (67.5%)***	16 (32.7%)***
T2DM	94 (59.9%)	74 (68.5%)**	20 (40.8%)**
Hypertension	140 (89.2%)	101 (93.5%)*	39 (79.6%)*
Dyslipidemia	128 (81.5%)	94 (87.0%)*	34 (69.4%)*
ASM	19.8 (16.9-23.2)	20.6 (18.1-24.3)***	17.1 (14.8-20.5)***
SMI	24.8 (22.3-27.3)	25.4 (22.3-27.6)	24.6 (22.2-27.2)
SI	0.634 (0.523-0.763)	0.668 (0.516-0.785)	0.607 (0.528-0.707)
Chair stand in seconds	10.8 (9.0-13.1)	11.1 (9.1-13.5)	10 (8.7-12.8)
SPPB	11 (9-12)	11 (9-12)	11 (9.3-12)
Low SMI	104 (66.2%)	81 (75.0%)***	23 (46.9%)***
Low SI	79 (50.3%)	58 (53.7%)	21 (42.9%)
Low muscle strength	18 (11.5%)	17 (15.7%)	2 (4.1%)
Poor muscle performance	26 (16.6%)	21 (19.4%)	6 (12.2%)
Sarcopenia using SMI	15 (9.6%)	14 (13.0%)*	1 (2.0%)*
Sarcopenia using SI	12 (7.6%)	10 (9.3%)	2 (4.1%)

Data presented as mean±SD, number (proportion), or median (interquartile range).
ASM: appendicular skeletal mass; BMI: Body Mass Index; NAFLD: non-alcoholic fatty liver disease; SI: Sarcopenic Index; SMI: Skeletal Mass Index; SPPB: short physical performance battery; T2DM: type 2 diabetes mellitus.
*P<0.05; **P<0.01; ***P<0.001.

TABLE II.—*Multivariable association of low SMI and NAFLD.*

Parameter	Model 1	Model 2	Model 3
Low SMI	3.0 (1.4-6.3)**	2.4 (1.1-5.2)*	1.2 (0.5-3.1)
Age	1.0 (1.0-1.0)	1.0 (0.9-1.0)	1.0 (0.9-1.0)
Sex, female	0.6 (0.3-1.3)	0.7 (0.3-1.5)	1.1 (0.4-2.9)
T2DM	-	2.1 (1.0-4.7)	2.5 (1.0-6.2)*
Hypertension	-	2.6 (0.8-8.7)	1.7 (0.4-6.5)
Dyslipidemia	-	1.6 (0.6-4.3)	1.4 (0.4-4.1)
BMI	-	-	1.0 (0.8-1.1)
Waist circumference	-	-	1.1 (1.0-1.2)**

BMI: Body Mass Index; T2DM: type 2 diabetes mellitus; Model 1: age and gender; model 2: model 1; T2DM; hypertension, and dyslipidemia; model 3: model 2, BMI and waist circumference.
*P<0.05; **P<0.01.

Discussion

In our study, we found an association of low SMI with NAFLD that was independent of demographic variables and comorbidities, but not of BMI and waist circumference.

Most studies have found an association of low SMI, as ASM/weight, and the presence of NAFLD.[25-29] However, all these studies were of Asian populations while our population was strictly Caucasian. In the current literature, of studies in a western setting, the association of low SMI and NAFLD was only found in sex-based subgroup analysis, with contradictory findings.[30, 31] Asian populations have different body composition that Caucasians, with increased abdominal fat deposition.[32] Genetic polymorphisms and lifestyle factors may also play an significant role in both NAFLD and sarcopenia pathophysiology.[1] As such, it is important these associations be tested in other ethnic populations.

Of previously published studies, only one accounted for BMI[33] and another for BMI and waist circumference.[34] The first did not find an association between SMI and NAFLD while

TABLE III.—*Multivariable association of sarcopenia (using SMI) and NAFLD.*

Parameter	Model 1	Model 2	Model 3
Sarcopenia using SMI	7.3 (0.9-58.9)	5.7 (0.7-48.1)	3.9 (0.4-33.0)
Age	1.0 (0.97-1.1)	1.0 (0.9-1.0)	1.0 (0.9-1.0)
Sex, female	0.48 (0.24-0.97)*	0.6 (0.3-1.2)	1.0 (0.4-2.8)
T2DM		2.0 (0.9-4.4)	2.3 (0.9-5.6)
Hypertension		2.8 (0.8-9.5)	1.6 (0.4-6.2)
Dyslipidemia		2.0 (0.7-5.2)	1.5 (0.5-4.7)
BMI			1.0 (0.8-1.1)
Waist circumference			1.1 (1.0-1.2)**

BMI: Body Mass Index; T2DM: type 2 diabetes mellitus; Model 1: age and gender; model 2: model 1; T2DM: hypertension, and dyslipidemia; model 3: model 2, BMI and waist circumference.
*P<0.05; **P<0.01.

TABLE IV.—*Characteristics of the population with NAFLD divided by presence of significant fibrosis.*

Parameter	All with NAFLD (N.=108)	With significant fibrosis by NFS (N.=17)	Without significant fibrosis by NFS (N.=91)
Sex, female	50 (46.3%)	8 (47.1%)	42 (46.2%)
Age	67 (59-71)	71 (65.5-72.5)**	64 (58-70)**
BMI	32.5 (29.2-36.0)	35.5 (32.6-45.0)**	32.1 (28.7-35.9)**
Waist circumference	110.3±11.4	115.2±11.1	109.4±11.3
Fat percentage	39.7 (34.8-47.0)	44.6 (35.1-51.0)	39.4 (34.7-46.7)
Obesity	73 (67.5%)	16 (94.1%)*	57 (62.6%)*
T2DM	74 (68.5%)	16 (94.1%)*	58 (63.7%)*
Hypertension	101 (93.5%)	17 (100%)	84 (92.3%)
Dyslipidemia	94 (87.0%)	16 (94.1%)	78 (85.7%)
ASM	20.6 (18.1-24.3)	21.5 (19.3-24.0)	20.2 (17.9-24.5)
SMI	25.4 (22.3-27.6)	23.2 (21.7-26.0)	25.8 (22.7-27.9)
SI	0.668 (0.516-0.785)	0.625 (0.470-0.704)	0.674 (0.543-0.797)
Chair stand in seconds	11.1 (9.1-13.5)	12.7 (9.9-16.8)	11.0 (9.0-12.8)
SPPB	11 (9-12)	10 (7-11)*	11 (10-12)*
Low SMI	81 (75.0%)	17 (100%)**	64 (70.3%)**
Low SI	58 (53.7%)	15 (88.2%)**	43 (47.3%)**
Low muscle strength	17 (15.7%)	5 (29.4%)	12 (13.2%)
Poor muscle performance	21 (19.4%)	5 (29.4%)	15 (16.5%)
Sarcopenia using SMI	14 (13.0%)	5 (29.4%) *	9 (9.9%) *
Sarcopenia using SI	10 (9.3%)	4 (23.5%) *	6 (6.6%) *

ASM: appendicular skeletal mass, BMI: Body Mass Index; NAFLD: non-alcoholic fatty liver disease; SI: Sarcopenic Index; SMI: Skeletal Mass Index; SPPB: short physical performance battery; T2DM: type 2 diabetes mellitus.
*P<0.05; **P<0.01.

the latter found an association in men but not in women. NAFLD is characteristically associated with visceral (abdominal) fat deposition as opposed to subcutaneous fat deposition, a pattern more common in men than women.[35] Adjustments for BMI, and particularly for waist circumference, are valuable in helping discern the relative weight of fat, fat distribution, and muscle on the outcome measured.

There was no association of muscle strength or muscle performance with NAFLD in our population. Most studies looking at muscle strength and NAFLD used handgrip, knee extension, or elbow flexor strength, with an inverse relationship being found in those that used weight or BMI adjusted measures[28, 36-41] but less consistently for those that used these measures unadjusted.[30, 31, 42] To the best of our knowledge, this was the first study to analyze strength as measured by the chair stand test in NAFLD. This test has been found to be an accurate surrogate for direct strength measurements.[22] It has the advantage of not requiring additional tools, making it low cost and easy to apply in the office, and enabling the replication of this study in more settings.

Sarcopenia, using SMI, was associated with NAFLD in univariable but not in multivariable analysis. However, given the low incidence of sarcopenia, an effect might have been lost. Few studies have looked at this association, with differing results and wide variation in methods of assessment of muscle mass and muscle strength.[30, 43, 44]

Limitations of the study

Our study had some limitations. The small population size did not allow for a multivariable analysis regarding the association of muscle parameters and fibrosis. This is unfortunate as univariable analysis showed lower SMI and SI and higher rates of sarcopenia in patients with advanced fibrosis. Low muscle mass is a feature of cirrhosis and has been associated with increased mortality in that population.[17] In previous studies, low SMI has been associated with fibrosis.[25, 33, 45, 46] It would be of great clinical relevance if low muscle mass was found to be a marker for significant, but possibly reversible, fibrosis before the development of overt, and usually irreversible, cirrhosis. Also, the methods chosen for determining fibrosis were non-invasive tools, as more accurate methods, like vibration-controlled transient elastography, were not readily available at our center at the time of this study.

Another limitation was the high percentage of NAFLD (68.8%) that was found. This may represent a selection bias: the population analyzed was comprised of patients with metabolic syndrome that were followed in a hospital clinic, which may suggest both a more serious disease burden and a more intensive treatment and follow-up. In the literature, most of the studies on this subject evaluated large populations of healthy cohorts;[25-27, 29, 40] our selection of patients with metabolic syndrome might have obfuscated some connections of muscle, liver and dysmetabolism.

A significant strength of our work was the careful definition of sarcopenia and its parameters, as recommended by expert consensus.[22] Within this subject, the use of inappropriate terminology and differing methodology is common. This hinders potential scientific progress by limiting not only comparison between studies, but also their replication, an important part of scientific production.

Conclusions

In conclusion, our study suggests that associations of low muscle mass and NAFLD may be related to other body composition differences that have not been accurately studied in previous works. The interplay of liver, fat, and muscle needs to be more thoroughly assessed, ideally expanding beyond cohort studies to mechanistic ones.

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Authors' contributions.—Joana Rigor was responsible for the design of the work, data acquisition and analysis, and drafting of the paper. Raquel Vasconcelos, Rogério Lopes, and Teresa Moreira made important contributions to acquisition and analysis of data and revised the paper critically. Pedro Barata and Daniela Martins-Mendes made substantial contributions to the design of the work and revised it critically. All authors approved the final version of the paper and agreed to be accountable for all aspects of the work.

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7. Fibroblast growth factor 21 and myostatin are higher in females with NAFLD and correlate with dysmetabolism and lower muscle mass, strength, and performance

7.1. Material and Methods

7.1.1. Population selection

Patients with MetS, aged 18 to 75 years-old, from an Internal Medicine outpatient clinic in a Portuguese tertiary hospital, were consecutively included. MetS was defined as the presence of at least three of the following five criteria: 1) waist circumference ≥102 cm for men or ≥88 cm for women; 2) elevated fasting plasma glucose (≥110 mg/dl), or known history or treatment of T2DM; 3) elevated blood pressure (≥130/85 mmHg), or history or treatment of HT; 4) low HDL-c <40 mg/dl for men or <50 mg/dl for women, or treatment for low HDL-c; 5) elevated TG (≥150 mg/dl) or treatment for elevated TG. Exclusion criteria were daily alcohol use >30 g in men and >20 g in women, clinically evident cirrhosis, liver disease of other etiology, chronic kidney disease stage ≥4, type 1 diabetes mellitus, hepatitis B, hepatitis C or HIV infection, active cancer, autoimmune disorders, major amputation, and no independent walking ability. Data regarding comorbidities, medication (including over-the-counter and supplements), and exercise habits, were collected by clinical interview, and medical file consultation when needed. The study was authorized by the hospital's Ethics Committee and every patient signed an informed consent before inclusion. Patient inclusion flow-chart can be seen in Figure 2.

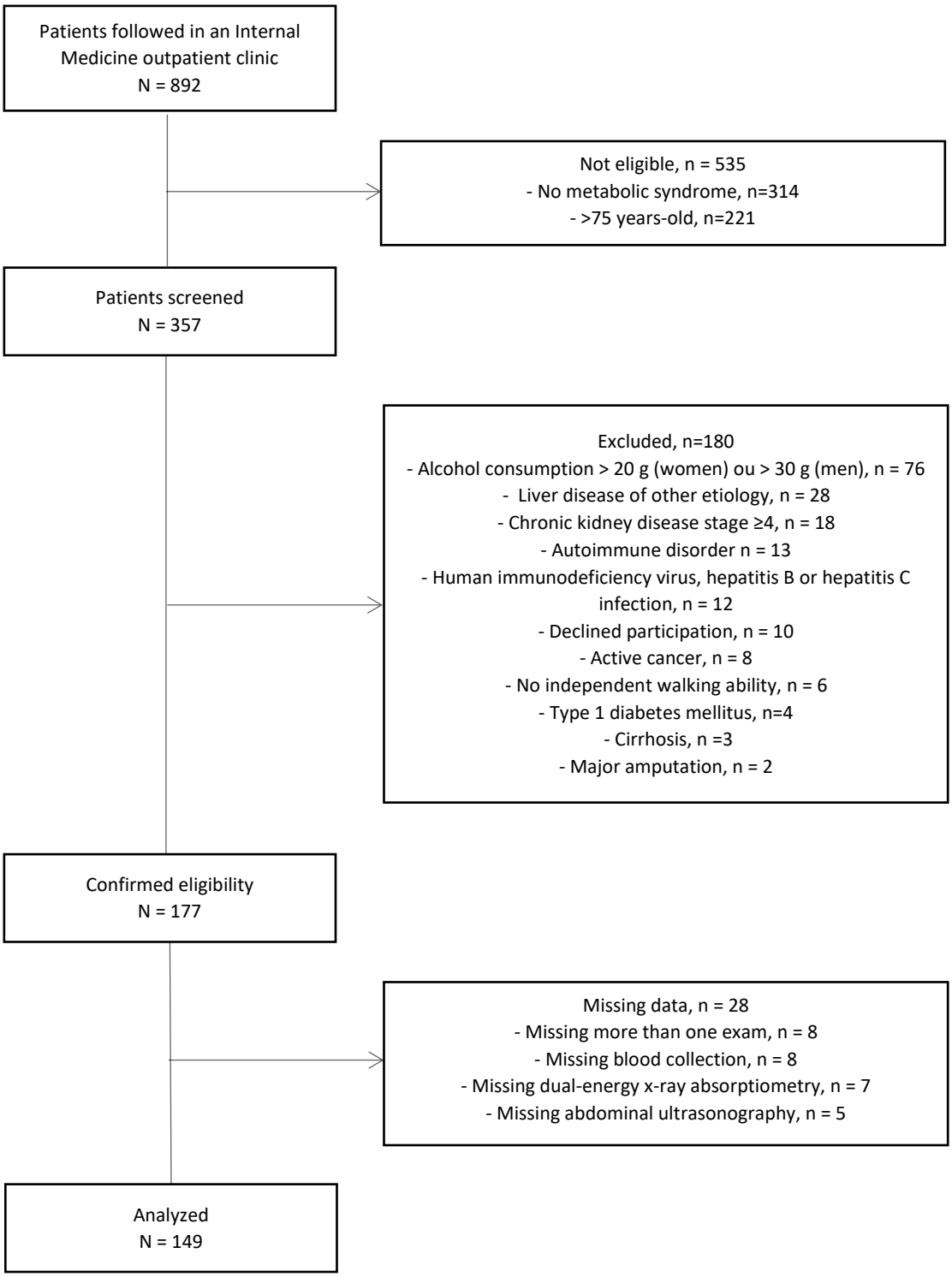

Figure 2 – Patient inclusion flow-chart

7.1.2. Anthropometric and muscle mass, strength, and physical performance assessment

Physical exam included weight, height, blood pressure, heart rate, and the SPPB. Gait speed and chair stand time were also analyzed independently of SPPB. BMI was calculated by dividing weight by height^2. ASM was determined by DXA and was used to calculate the skeletal mass index (SMI = ASM / weight * 100, in %) and the sarcopenia index (SI = ASM / BMI), as per recommendations.(7) Body fat percentage (as % of body weight) was also measured by DXA.

7.1.3. NAFLD diagnosis

NAFLD was diagnosed by abdominal ultrasonography by an experienced radiologist. Other causes of hepatic disease were excluded, including alcoholic liver disease, autoimmune, metabolic, infiltrative, and neoplastic, by careful history taking, physical examination, and additional blood test and imaging, as necessary.

7.1.4. Laboratory methods

Venous blood was collected after at least 8h of no food or strenuous exercise. Serum lipids (including total cholesterol, HDL-c, and TG), fasting plasma glucose, fasting insulin, and HbA1c were measured by automated methods. HOMA-IR was calculated as insulin (mg/dL) multiplied by fasting plasma glucose (mg/dL) and divided by 405. Serum was centrifuged and stored at -80ºC until time of measurement of myokines and adipokines. Commercially available enzyme-linked immunosorbent assay (ELISA) kits from DLdevelop (Wuxi Donglin Sci & Tech Development Co., Ltd., Wuxi, Jiangsu, China) for FNDC5, myonectin, and myostatin, DRG Diagnostics (DRG Instruments GmbH, Marburg, Germany) for FGF21 and leptin, Invitrogen (Thermo Fisher Scientific, MA, USA) for IL-6, and Proteintech (Proteintech Group, Inc, IL, USA) for adiponectin were used according to manufacturer instructions.

7.1.5. Statistical analysis

Continuous variables were described as mean ± standard deviation (SD) if they had normal distribution, and median and interquartile range (IQR) if not. Categorical variables were presented as absolute number and frequencies. For univariate analysis, Student t test for independent samples or Mann-Whitney U test were used, according

to the variable's distribution. Spearman's rank correlation was used to assess the relationship between each adipokine and myokine (adiponectin, leptin, FGF21, IL-6, FNDC5, myonectin, and myostatin) and metabolic factors (HbA1c, HOMA-IR, TG, TC, HDL-c, TC/HDL-c ratio) and body composition parameters (BMI, body fat, SMI, gait speed, chair stand time and SPPB). P value < 0.05 was considered significant. IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp, Armonk, NY) was used for all statistical analyses.

7.2. Results

7.2.1. Demographic characteristics

A total of 149 patients were included, 50.3% male, with a median age of 67 years (IQR 61 - 71). All patients were white. The prevalence of NAFLD was 73.2% (82.7% in males and 63.5% in females, $p < 0.05$). All females were post-menopausal (minimum time from menopause of 4 years). Table 8 presents the demographic characteristics of the population studied, divided by sex, and subdivided by presence or absence of NAFLD. In both females and males, patients with NAFLD had higher BMI and higher body fat percentage. In females, NAFLD was also accompanied by a higher prevalence of T2DM (70.2% vs. 25.9%, $p < 0.001$), higher HbA1c (6.6% vs. 5.9%, $p < 0.001$), higher HOMA-IR (4.9 vs. 2.0, $p < 0.001$), higher TG (130 mg/dl vs. 93.0 mg/dl, $p < 0.01$), and lower HDL-c (48.0 mg/dl vs. 57.7 mg/dl, $p < 0.001$).

Females with NAFLD had lower SMI (22.2% vs. 23.3%, $p < 0.05$), longer chair stand time (11.9 s vs. 10.2 s, $p < 0.05$), slower gait speed (0.6 m/s vs. 0.7 m/s, $p < 0.05$), and lower SPPB (11.0 vs. 12.0, $p < 0.01$).

Regarding myokines and adipokines, males with NAFLD had lower levels of leptin (4.3 ng/ml vs. 5.0 ng/ml, $p < 0.05$) than those without, while females with NAFLD had higher levels of FGF21 (259.0 ng/ml vs. 84.0 ng/ml, $p < 0.01$) and myostatin (2.5 ng/ml vs. 2.4 ng/ml, $p < 0.05$).

Table 8 – Characteristics of the population divided by sex and the presence of NAFLD

	Male			Female		
	All (n=75)	With NAFLD (n=62)	Without NAFLD (n=13)	All (n=74)	With NAFLD (n=47)	Without NAFLD (n=27)
Age (years), median (IQR)	67.0 (61.0-71.0)	67.0 (59.8-71.0)	68 (66.5-71.5)	65.5 (58.8-71.0)	67.0 (59.0-71.0)	64.0 (58.0-71.0
T2DM, n (%)	55 (73.3%)*	44 (71.0%)	11 (84.6%)	40 (54.1%)*	33 (70.2%)***	7 (25.9%)***
HbA1c (%), median (IQR)	6.7 (6.0-7.5)	6.6 (6.0-7.6)	6.7 (6.2-7.5)	6.3 (5.9-7.5)	6.6 (6.2-8.0)***	5.9 (5.6-6.6)***
HOMA-IR, median (IQR)	3.1 (1.9-4.9)	3.1 (1.7-4.9)	2.5 (2.0-4.6)	2.9 (1.9-6.1)	4.9 (2.6-7.2)***	2.0 (1.4-2.9)***
TG (mg/dl), median (IQR)	134.0 (93.0-181.0)	137.0 (105.0-189.0)	114.0 (76.5-149.5)	124.0 (91.3-156.5)	130.0 (104.0-170.0)**	93.0 (75.0-147.0)**
TC (mg/dl), mean ± SD	160.8 ± 39.7	165.1 ± 41.0*	140.2 ± 24.2*	171.9 ± 33.5	165.3 ± 31.9 **	183.4 ± 33.8**
HDL-c (mg/dl), mean ± SD	44.8 ± 10.9***	45.1 ± 11.6	43.2 ± 7.3	51.5 ± 10.6***	48.0 ± 9.6***	57.7 ± 9.6***
TC/HDL-c ratio, median (IQR)	3.5 (2.9-4.2)	3.7 (2.9-4.2)	3.1 (2.7-3.5)	3.3 (2.8-3.9)	3.3 (2.9-4.1)	3.1 (2.7-3.7)
Obesity, n (%)	36 (48.0%)	34 (54.8%) **	2 (15.4%) **	46 (62.2%)	38 (80.9%) ***	8 (29.6%) ***
BMI (kg/m^2), median (IQR)	29.8 (27.4-32.8)*	30.7 (27.5-33.6)*	28.1 (25.0-29.5)*	32.5 (27.8-37.4)*	34.3 (30.9-39.7)***	27.7 (25.9-32.3)***
Body fat (%), mean ± SD	33.8 ± 5.6***	34.7 ± 5.1**	30.2 ± 6.6**	45.5 ± 4.9***	46.8 ± 4.6**	43.5 ± 4.8**
SMI (%), mean ± SD	27.4 ± 2.1***	27.3 ± 1.8	28.1 ± 2.9	22.6 ± 2.1***	22.2 ± 2.1*	23.3 ± 2.0*
SI, mean ± SD	0.8 ± 0.1***	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.1***	0.5 ± 0.1	0.6 ± 0.1
Chair stand (s), median (IQR)	10.4 (8.8-12.8)*	10.3 (8.5-12.4)	10.8 (9.7-14.2)	11.2 (9.9-13.2)*	11.9 (10.3-14.5)**	10.2 (9.0-11.3)**
Gait speed (m/s), mean ± SD	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1*	0.7 ± 0.1*

SPPB, median (IQR)	11.0 (9.0-12.0)	11.0 (10.0-12.0)	10 (8.5-11.5)	11.0 (9.0-12.0)	11.0 (8.0-11.0)**	12 (10.5-12-0)**
Adiponectin (pg/ml), median (IQR)	1255.7 (599.3-1771.0)	1247.9 (576.5-1731.3)	1491.7 (679.2-1848.2)	1436.0 (913.4-2049.0)	1416.3 (890.8-2080.1)	1456.4 (1046.1-1978.5)
FGF21 (pg/ml), median (IQR)	203.1 (79.1-412.0)	214.1 (81.8-387.7)	167.9 (60.1-460.3)	215.2 (98.3-397.5)	259.0 (140.0-429.0)**	84.0 (49.8-306.2)**
FNDC5 (pg/ml), median (IQR)	3.1 (1.0-5.6)*	3.1 (1.0-5.4)	2.8 (1.0-7.4)	3.8 (2.0-9.0)*	3.3 (0.8-8.9)	5.8 (3.1-9.1)
IL-6 (pg/ml), median (IQR)	1.3 (1.1-1.8)	1.4 (1.1-1.9)	1.2 (1.0-1.7)	0.7 (0.6-0.8)	1.5 (1.0-1.9)	1.3 (1.0-1.5)
Leptin (ng/ml), median (IQR)	4.3 (2.0-8.3)***	5.0 (2.6-9.8)*	2.0 (1.1-4.9)*	11.3 (5.9-21.1) ***	14.3 (7.8-22.8)	8.1 (5.2-16.8)
Myonectin (ng/ml), median (IQR)	0.2 (0.2-0.4)	0.2 (0.2-0.4)	0.2 (0.2-0.5)	0.3 (0.2-0.4)	0.3 (0.3-0.5)	0.2 (0.1-0.4)
Myostatin (ng/ml), median (IQR)	3.2 (2.1-8.1)**	3.1 (2.1-7.7)	4.2 (1.9-10.9)	2.3 (1.9-3.7)**	2.5 (2.0-6.4)*	2.4 (1.9-3.9)*

BMI – body mass index, FGF21 – fibroblast growth factor 21, FNDC5 – fibronectin type III domain-containing protein 5, HbA1c – glycated hemoglobin, HDL-c – high-density lipoprotein, HOMA-IR – homeostasis model assessment for insulin resistance, IL-6 – interleukine-6, IQR – interquartile range, NAFLD – non-alcoholic fatty liver disease, SD – standard deviation, SI – sarcopenic index, SMI – skeletal mass index, SPPB – short physical performance battery, T2DM – type 2 diabetes mellitus, TC – total cholesterol, TG – triglycerides. *p<0.05, **p<0.01, ***p<0.001

7.2.2. Myokines, adipokines, and metabolic parameters

Table 9 describes the Spearman's rank correlation between the myokines and adipokines and HbA1c, HOMA-IR, TG, TC, HDL-c, and TC/HDL-c ratio, divided by sex.

In both males and females, leptin was positively correlated with HOMA-IR (ρ = 0.305 in males and ρ = 0.368 in females, $p < 0.01$); in females, leptin was also positively correlated with TG (ρ = 0.240, $p < 0.05$) and negatively correlated with HDL-c (ρ = 0.287, $p < 0.05$). Adiponectin had metabolic correlations in males but not in females; negative correlations were found with HOMA-IR (ρ = -0.242, $p < 0.05$) and TC/HDL-c ratio (ρ = -0.235, $p < 0.05$). In males, FNDC5 was negatively correlated (ρ = -0.238, $p < 0.05$) while IL-6 was positively correlated with TG (ρ = 0.233, $p < 0.05$), and myonectin was positively correlated with HOMA-IR (ρ = 0.261, $p < 0.05$). In females, myostatin was negatively correlated with TC (ρ = -0.294, $p < 0.05$).

There were several findings regarding FGF21. In both sexes, it was positively correlated with TG (ρ = 0.321, $p < 0.01$, in males; ρ = 0.389, $p < 0.001$, in females) and TC/HDL-c ratio (ρ = 0.233 in males and 0.264 in females, $p < 0.05$), and negatively correlated with HDL-c (ρ = -0.233, $p < 0.05$, in males; ρ = -0.484, $p < 0.001$, in females). Additionally, in females, FGF21 was positively correlated with HbA1c (ρ = 0.381, $p < 0.001$) and HOMA-IR (ρ = 0.404, $p < 0.001$).

Table 9. Spearman's rank correlation between myokines and adipokines and metabolic parameters

Male						
	HbA1c	HOMA-IR	TG	TC	HDL-c	TC/HDL-c ratio
Adiponectin	-0.027	-0.242*	-0.106	-0.118	0.132	-0.235*
FGF21	0.193	0.203	0.321**	-0.009	-0.233*	0.233*
FNDC5	-0.160	-0.016	-0.238*	-0.099	0.005	-0.088
IL-6	0.129	0.136	0.233*	0.014	-0.100	-0.083
Leptin	-0.019	0.305**	0.093	-0.029	-0.092	0.021
Myonectin	0.060	0.261*	0.214	-0.100	-0.165	0.059
Myostatin	0.103	-0.099	0.128	-0.099	-0.066	0.021
Female						
	HbA1c	HOMA-IR	TG	TC	HDL-c	TC/HDL-c ratio
Adiponectin	-0.077	-0.023	-0.002	0.063	0.172	-0.137
FGF21	0.381***	0.404***	0.389***	-0.197	-0.484***	0.264*
FNDC5	-0.025	-0.196	-0.197	0.022	0.128	-0.126
IL-6	0.115	0.231	0.129	-0.067	-0.149	0.095
Leptin	0.174	0.368**	0.240*	-0.131	-0.287*	0.163
Myonectin	-0.042	0.174	0.084	0.126	-0.145	0.229
Myostatin	0.281	0.115	0.044	-0.294*	-0.161	-0.118

BMI – body mass index, FGF21 – fibroblast growth factor 21, FNDC5 – fibronectin type III domain-containing protein 5, HbA1c – glycated hemoglobin, HDL-c – high-density lipoprotein, HOMA-IR – homeostasis model assessment for insulin resistance, IL-6 – interleukine-6, NAFLD – non-alcoholic fatty liver disease, T2DM – type 2 diabetes mellitus, TC – total cholesterol, TG – triglycerides. *p<0.05, **p<0.01, ***p<0.001

7.2.3. Myokines, adipokines, BMI and muscle parameters

The Spearman's rank correlations for myokines/adipokines and BMI, SMI, gait speed, chair stand time, and SPPB are shown in Table 10, divided by sex.

In females and males alike, leptin was positively correlated with BMI ($\rho = 0.533$ in males and $\rho = 0.532$ in females, $p < 0.001$) and body fat ($\rho = 0.586$ in males and $\rho = 0.438$ in females, $p < 0.001$), and negatively correlated with SMI ($\rho = -0.361$ in males and $\rho = -0.325$ in females, $p < 0.01$). In females, leptin was also positively correlated with

chair stand time (ρ = 0.306, p < 0.01). In males, negative correlations were found between myonectin and gait speed (ρ = -0.240, p < 0.05), and between myostatin and BMI (ρ = -0.258, p < 0.05).

In females only, myostatin was correlated with all muscle parameters, with a negative correlation with SMI (ρ = -0.240, p < 0.05), gait speed (ρ = 0.261, p < 0.05), and SPPB (ρ = 0.248, p < 0.05), and a positive correlation with chair stand time (ρ = 0.259, p < 0.05). Similarly, FGF21 was negatively correlated with SMI (ρ = 0.330, p < 0.01), gait speed (ρ = -0.247, p < 0.05), and SPPB (ρ = -0.326, p < 0.01); it was also positively correlated with BMI (ρ = 0.272, p < 0.05) and body fat (ρ = 0.352, p < 0.05).

Table 10. Spearman's rank correlation between myokines and adipokines, and body composition and muscle parameters

Male						
	BMI	Body fat	SMI	Gait speed	Chair stand time	SPPB
Adiponectin	0.015	-0.138	-0.143	-0.047	0.029	-0.017
FGF21	-0.010	0.032	-0.117	-0.074	-0.032	0.001
FNDC5	0.116	0.185	-0.128	0.101	-0.063	0.115
IL-6	0.153	0.241	-0.182	-0.017	-0.121	0.125
Leptin	0.533***	0.586***	-0.361**	0.125	-0.064	0.206
Myonectin	0.079	-0.046	0.032	-0.240*	-0.096	-0.072
Myostatin	-0.258*	-0.200	0.013	-0.110	0.192	-0.213
Female						
	BMI	Body fat	SMI	Gait speed	Chair stand time	SPPB
Adiponectin	-0.096	-0.138	0.174	0.180	-0.1551	0.083
FGF21	0.272*	0.352**	-0.330**	-0.247*	0.190	-0.326**
FNDC5	-0.181	-0.203	0.195	-0.008	0.118	-0.209
IL-6	0.189	0.181	-0.175	0.074	0.109	-0.128
Leptin	0.532***	0.438***	-0.325**	-0.165	0.306**	-0.144
Myonectin	0.120	0.091	-0.109	-0.066	-0.070	0.053
Myostatin	0.161	0.191	-0.240*	-0.261*	0.259*	-0.248*

BMI – body mass index, FGF21 – fibroblast growth factor 21, FNDC5 – fibronectin type III domain-containing protein 5, IL-6 – interleukine-6, SMI – skeletal mass index, SPPB – short physical performance battery. *p<0.05, **p<0.01, ***p<0.001

7.3. Discussion

Our study revealed different body composition, metabolic, and biochemical patterns associated with NAFLD, according to sex. We also found associations between these different patterns.

Prevalence of NAFLD was higher in men than in women. Female, but not male, patients with NAFLD presented more metabolic dysfunction than their counterparts without NAFLD, mainly with higher prevalence of T2DM, higher levels of HbA1c%, HOMA-IR, and TG, and lower levels of HDL-c. Previous studies have found that male sex is a risk factor for NAFLD independent of metabolic factors.(164) In postmenopausal females, increased risk for NAFLD is dependent on metabolic factors, such as metabolic syndrome, obesity, weight gain, and insulin resistance.(164) This suggests the "hits" involved in the pathophysiology of NAFLD may be different in females and males.

Differences in body composition between men and women and between premenopausal and postmenopausal women have been suggested as a driver for these sex disparities. In our study, while increased BMI and body fat percentage was found in patients with NAFLD in both males and females, a lower SMI in patients with NAFLD was only found in females. Currently, there is conflicting evidence regarding the importance of sex in the association between muscle mass and NAFLD, with some authors finding an association in females but not males,(114, 132) and others the opposite.(102, 110, 115, 117, 127) Of note, most of these studies were conducted in Asian populations which present different body composition, with decreased muscle mass for the same BMI, when compared to other ethnicities.(163)

Levels of leptin and FNDC5 were higher in females, while levels of myostatin were higher in males, which is consistent with previous studies, although evidence is scarce.(165-168) Patterns of myokines according to NAFLD were different for males and females, with males with NAFLD having higher leptin and females with NAFLD having higher FGF21 and myostatin.

Levels of leptin have been associated with NAFLD in meta-analyses,(89) but, in our population, this association was only identified in males. Leptin is produced predominantly in subcutaneous adipocytes as opposed to visceral adipocytes, and tissue

adipose distribution is different according to sex.(169) While leptin is responsible for reducing appetite and inhibiting the synthesis of lipids in both sexes, males are more susceptible to leptin resistance.(170) In our study, leptin was positively correlated with HOMA-IR, BMI, and body fat, and negatively correlated with SMI, which is consistent with its production and the metabolic effects described above.(169)

FGF21 was correlated with an unfavorable lipid profile and, in females, with insulin resistance as well. In females, FGF21 was also correlated with higher BMI, higher body fat, lower muscle mass and lower physical performance. In the literature, higher levels of FGF21 have been found to be associated with lower HDL-c, higher TC, and higher TG,(80) and with the presence of NAFLD.(79) In animal models, FGF21 has been shown to be required for fasting-induced muscle loss and weakness.(77) Given our finding of increased prevalence of metabolic risk factors in females, but not males, with NAFLD, these findings suggest a role of FGF21 in the association between dysmetabolism and NAFLD.

In the literature, results regarding myostatin are conflicting with some studies reporting a positive association with muscle mass in males but not females,(70) others a positive association with sarcopenia in males,(69, 171) another an inverse relationship with strength in females but not in males,(68) and another a direct association with strength in males but not females and better physical performance in both sexes.(67) In our population, in females, higher myostatin was correlated with lower muscle mass, strength, and performance. Myostatin was also higher in females with NAFLD. There were no correlations of myostatin with BMI, body fat, insulin resistance, and lipids. Since, in females, there was also a relationship between worse muscle parameters and NAFLD, myostatin may be a possible link between these variables.

Interestingly, in our population, adiponectin was not significantly lower in patients with NAFLD, as it has been found in the literature.(172) Moreover, it was also not negatively correlated with BMI or body fat, as it has been well established.(173) In our sample, adiponectin concentrations were lower than usually found.(174) These findings may be a result of population selection bias: since the presence of MetS was an inclusion criterion, most patients were obese; as adiponectin expression is

downregulated in obesity, serum levels are overall lower making statistically significant differences harder to detect.

One of the limitations of this study was the high prevalence of NAFLD. Prevalence has been found to be around 25% in the general population and 50% of those with obesity or T2DM. In our population, prevalence was 73.2% (82.7% in males). Patients with MetS warranting follow-up in a hospital outpatient clinic usually have more difficult to control or advanced disease than patients in a primary care setting, which could justify the high prevalence of NAFLD. As it is known, very high prevalence of outcomes may pose a statistical obstacle for association recognition.

Sarcopenia is a disorder commonly associated with aging and the elderly, with increasing prevalence with age.(7) Although we excluded patients older than 75 years old, our population was still elderly, with a median age of 67 years (IQR 61-71). It would be important to analyze the associations shown in our study in younger populations, as they are more likely to develop the negative outcomes associated with NAFLD.

Our study was single center and the population included represented a very homogenous ethnic group. This represents a limitation and should be considered when interpreting our results. Body composition varies significantly according to race,(163) and so does the prevalence and natural history of NAFLD.(1)

7.4. Conclusion

In our population, there was a sex difference in metabolic and muscle parameters associated with NAFLD, with females with NAFLD presenting with more dysmetabolism and worse muscle parameters. Patterns of myokines analyzed also varied according to sex, with females with NAFLD having higher FGF21 and higher myostatin. In females, FGF21 was correlated with worse metabolic parameters, while myostatin was correlated with worse muscle parameters, suggesting that FGF21 is a possible link between NALFD and dysmetabolism, and myostatin between NAFLD and muscle health. Going forward, mechanistic and longitudinal studies are needed to determine the exact influence of muscle and myokines in NAFLD.

8. Gut microbiome composition and its associations with NAFLD and low muscle mass

8.1. Material and Methods

8.1.1. Population selection

Thirty patients with NAFLD and thirty patients without NAFLD were randomly selected from an outpatient MetS cohort. Inclusion criteria were age 18 to 75 years-old and the presence of MetS, defined as the presence of three or more of the following five criteria: 1) waist circumference ≥102 cm for men or ≥88 cm for women; 2) elevated fasting plasma glucose (≥110 mg/dl), or known history or treatment of T2DM; 3) elevated blood pressure (≥130/85 mmHg), or history or treatment of HT; 4) low HDL-c <40 mg/dl for men or <50 mg/dl for women, or treatment for low HDL-c; 5) elevated TG (≥150 mg/dl) or treatment for elevated TG. Exclusion criteria were alcohol use >30 g in men and >20 g in women, clinically evident cirrhosis, liver disease of other etiology, chronic kidney disease stage ≥4, type 1 diabetes mellitus, infection by hepatitis B, hepatitis C or human immunodeficiency virus, active cancer, autoimmune disorders, major amputation, and no independent walking ability.

8.1.2. NAFLD diagnosis, biochemical characterization, and anthropometric and muscle mass, strength, and physical performance assessment

NAFLD was diagnosed with abdominal ultrasonography by an experienced radiologist. Other causes of hepatic disease were excluded, including alcoholic, autoimmune, infiltrative, and other metabolic causes. Venous blood was collected after at least 8h of no food or strenuous exercise. Plasma glucose, insulin, HbA1c, total cholesterol, HDL-c, and triglycerides were measured by automated methods. HOMA-IR was calculated as insulin (mg/dL) multiplied by plasma glucose (mg/dL) and divided by 405. BMI was obtained by weight (in kg) / height^2 (in m). SMI was calculated as ASM / body weight x 100, with ASM assessed by DXA. Low SMI was defined as <22.9% for females and <29.0% for males. DXA was also used to determine body fat percentage. The Short Physical Performance Battery (SPPB), a composite test of gait speed, balance, and lower body strength, was performed. Low muscle strength was defined has a chair stand test (time to rise from sitting 5 times) of > 15 s and low physical performance was defined has a SPPB ≤ 8.

8.1.3. Microbiome collection and analysis

Patients collected a faecal sample and sent it to the researchers. Results for each family of bacteria are given as a percentage in comparison with a healthy control group. Genomic DNA was extracted automatically using Lab-Aid 824s DNA Extraction Kit Handbook (Zeesan, China). DNA concentration and 260/280 ratio were obtained with µDrop™ Plate and Thermo Sicentific™ Multiskan SkyHigh Microplate Spectrophotometer. Samples were diluted to a concentration of 10 ng/µl. The amplification of the sequences of interest was performed with the NZYSpeedy qPCR Green Master Mix Kit (NZYtech – Genes & Enzymes) in qTOWER3 Real-Time PCR Thermal Cycler (Analytik jena) under the following conditions: incubation at 95º C for 3 minutes and 40 cycles of 95º C/5 s and 60º C/30 s. Eleven pairs of primers were analysed. Relative quantification was determined by 2^(-ΔΔC),(175) using ribosomal gene 16S as internal control.

8.1.4. Statistical analysis

Continuous variables with normal distribution were described as mean ± standard deviation (SD), continuous variables without normal distribution as median and interquartile range (IQR), and categorical variables as absolute number and frequencies. Mann-Whitney U test and Student t test were used for univariate analysis. All statistical analyses were performed in IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp, Armonk, NY).

8.2. Results

8.2.1. Demographic characteristics

In total, 48 patients were included, 24 with NALFD and 24 without. Population characteristics are expanded on in Table 11. Most patients were female (56.3%) and had T2DM (60.4%). Median age was 67.0 (60.0-70.8) years. Prevalence of low SMI was 60.4%, low muscle strength 10.4%, and low physical performance 18.8%. Patients with NAFLD had lower HDL-c (44.8 vs. 51.4 mg/dl, $p < 0.05$) and higher BMI (35.7 vs. 27.6 mg/dl, $p < 0.01$).

Table 11 – Characteristics of the population, divided by low or normal SMI

	All (n = 48)	With NAFLD (n = 24)	Without NAFLD (n = 24)
Age, median (IQR)	67.0 (60.0-70.8)	67.0 (59.0-71.5)	67.0 (63.0-70.5)
Female, n (%)	27 (56.3%)	11 (45.8%)	16 (66.7%)
T2DM, n (%)	29 (60.4%)	17 (70.8%)	14 (58.3%)
HbA1c (%), median (IQR)	6.4 (5.8-7.4)	6.4 (5.8-7.4)	6.5 (5.8-7.5)
HOMA-IR, median (IQR)	2.7 (1.5-4.9)	2.2 (1.6-4.2)	3.7 (1.4-6.2)
TG (mg/dl), median (IQR)	108.5 (85.8-144.3)	111.0 (95.0-143.8)	104.5 (79.8-150.3)
TC (mg/dl), mean ± SD	160.3 ± 40.2	157.0 ± 42.4	163.7 ± 38.5
HDL-c (mg/dl), mean ± SD	48.1 ± 10.7	44.8 ± 10.4*	51.4 ± 10.2*
BMI (kg/m^2), median (IQR)	30.4 (26.5-36.6)	35.7 (30.7-37.2)**	27.6 (25.4-30.0)**
Body fat (%), mean ± SD	40.1 ± 8.5	40.9 ± 8.1	39.3 ± 9.0
SMI (%), mean ± SD	24.8 ± 3.0	25.2 ± 3.1	24.4 ± 2.9
Chair stand time (s), mean ± SD	11.7 ± 2.3	12.3 ± 2.6	11.1 ± 1.8
Gait speed (s), mean ± SD	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
SPPB, median (IQR)	11.0 (9.0-12.0)	11.0 (8.0-12.0)	11.0 (9.0-12.0)
Low SMI, %	29 (60.4%)	17 (70.8%)	12 (50.0%)
Low muscle strength, %	5 (10.4%)	4 (16.7%)	1 (4.2%)
Poor physical performance, %	9 (18.8%)	7 (29.2%)	2 (8.3%)

BMI – body mass index, HbA1c – glycated hemoglobin, HDL-c – high-density lipoprotein, HOMA-IR – homeostasis model assessment for insulin resistance, IQR – interquartile range, NAFLD – non-alcoholic fatty liver disease, SD – standard deviation, SMI – skeletal mass index, SPPB – short physical performance battery, T2DM – type 2 diabetes mellitus, TC – total cholesterol, TG – triglycerides. *$p<0.05$, **$p<0.01$

8.2.2. Microbiome distribution according to NAFLD and muscle mass

In our population, compared to healthy adult controls, the relative quantity of *Actinobacteria*, *Atopobacter*, *Bacteroidetes*, *Bacteriodes fragilis*, *Bifidobacterium*, *Clostridium coccoides*, *Clostridium leptum*, *Firmicutes*, *Lactobacillus*, and *Prevotella* was decreased, while *Proteobacteria* was increased ($p < 0.05$). In Table 12, the distribution of microbiota population is described. There were no differences found when comparing patients with and patients without NAFLD. The groups were further divided in those with low SMI and those with normal SMI. In patients without NAFLD, those with low SMI had a smaller comparative abundance of *Bifidobacterium* (3.2% vs. 30.3%, $p < 0.05$). Analyses including low muscle strength and low physical performance were not performed given the small number of events.

Table 12 – Microbiota population distribution in low and normal SMI according to the presence or absence of NAFLD

	With NAFLD			Without NAFLD		
	All (n=24)	Low SMI (n=17)	Normal SMI (n=7)	All (n=24)	Low SMI (n=12)	Normal SMI (n=12)
Actinobacteria (%), mean (IQR)	26.4 (10.7-58.4)	26.8 (11.8-61.6)	18.9 (3.9-59.2)	17.1 (5.8-49.8)	11.3 (3.1-36.5)	33.0 (9.4-63.5)
Atopobacter (%), mean (IQR)	53.1 (24.1-153.9)	53.3 (24.2-208.3)	35.6 (23.5-54.6)	47.6 (26.9-79.9)	47.3 (25.3-71.3)	47.9 (33.9-118.1)
Bacteroidetes (%), mean (IQR)	42.5 (19.7-77.6)	35.7 (7.7-70.8)	46.6 (43.1-80.1)	54.0 (19.6-130.6)	48.1 (17.0-121.8)	54.0 (22.9-166.1)
Bacteroides fragilis (%), mean (IQR)	58.3 (11.8-141.3)	54.2 (9.5-135.0)	70.3 (13.3-350.9)	49.7 (16.2-224.0)	45.0 (16.2-169.2)	57.0 (13.8-249.1)
Bifidobacterium (%), mean (IQR)	20.5 (10.8-64.7)	21.6 (10.8-83.1)	19.6 (2.5-55.8)	14.7 (2.2-46.7)	3.2 (1.1-17.1)*	30.3 (14.5-62.6)*
Clostridium coccoides (%), mean (IQR)	93.9 (47.5-176.0)	95.4 (47.6-183.7)	92.4 (45.9-135.8)	61.5 (27.3-107.3)	50.3 (40.5-196.8)	67.0 (21.7-102.4)
Clostridium leptum (%), mean (IQR)	64.1 (41.6-179.5)	59.8 (41.9-176.4)	82.6 (35.2-185.7)	58.1 (34.2-92.8)	63.7 (31.5-137.4)	55.3 (34.9-75.4)
Firmicutes (%), mean (IQR)	74.5 (53.4-113.9)	70.2 (52.4-111.8)	86.2 (52.9-129.7)	64.4 (49.2-84.8)	64.4 (50.0-87.3)	66.2 (49.2-79.7)
Lactobacillus (%), mean (IQR)	44.4 (4.3-742.0)	92.0 (7.4-941.7)	15.3 (3.2-123.6)	27.8 (9.1-107.4)	20.5 (4.3-71.9)	33.1 (16.0-195.1)
Prevotela (%), mean (IQR)	4.9 (1.2-45.1)	5.3 (1.5-27.8)	4.5 (0.5-211.1)	11.1 (2.2-153.7)	10.4 (1.3-188.1)	13.8 (2.2-142.5)
Proteobacteria (%), mean (IQR)	465.8 (51.4-938.5)	520.4 (111.6-1610.9)	90.7 (9.4-780.7)	298.3 (71.1-771.3)	281.7 (74.7-724.2)	308.6 (65.4-1094.5)

IQR – interquartile range. *p<0.05

8.3. Discussion

In our population, there was no significant difference in gut microbiome population in patients with NAFLD compared to those without. In the literature, evidence is sometimes conflicting,(98) but, overall, there seems to be an increase in *Escherichia*, *Prevotella,* and *Streptococcus,* and decrease in *Bacteroides fragilis, Coprococcus*, *Clostridium coccoides, Faecalibacterium* and *Ruminococcus*.(14, 97) Most studies have been performed with healthy individuals as controls, while our control population was comprised of patients with MetS without NALFD, which differed in BMI and levels of HDL-c but had the same rates of T2DM and insulin resistance. This may justify the absence of significant differences. As in NAFLD, in T2DM, populations of *Escherichia* and *Prevotella* are increased,(95) and populations of *Lactobacillus* are decreased. (14) In obesity, *Clostridium*, *Lactobacillus,* and *Prevotella*, among others, are increased, *Bifidobacterium* is decreased, while evidence regarding *Bacterioidetes*, *Bacterioides*, and *Proteobacteria* is conflicting. (176)

In patients without NAFLD, there was a significantly larger decrease in *Bifidobacterium* in those with low SMI. However, when not considering this group, patients with NAFLD had a trend towards a lower relative abundance of this genus. This may suggest a relationship of *Bifidobacterium* in both NAFLD and muscle health. *Bifidobacterium* is a genus of gram-positive anaerobic bacteria that ferments carbohydrates. Beyond digestion, it has important immunologic functions: in mice, reduced numbers of *Bifidobacterium* have been shown to increase endotoxemia,(13) and, in humans, *Bifidobacterium* has been inversely associated with ferritin, a marker of inflammation. (177) As such, *Bifidobacterium* supplementation has been suggested as a treatment in obesity, T2DM, and NAFLD.(178) Regarding muscle health, athletes have been shown to have an increased relative abundance of this genus.(179)

Our major limitation in this study was a small population size, that prevented further analyses, namely associations with low muscle strength and low physical performance. In this field of study, data is scarce and mostly coming from animal studies. However, some of the most significant evidence has been concerning the positive association of *Bifidobacterium* with grip strength.(15) Given the association of this genus

with muscle mass in our population, an analysis of muscle strength would have been of particular interest.

8.4. Conclusion

In our study, *Bifidobacterium* was reduced in patients with low SMI and no NAFLD, and in patients with NAFLD with normal or low SMI comparing with patients with no NAFLD and normal SMI. This suggests a link between *Bifidobacterium* and low muscle mass. Our work was limited by a small sample size that may have obfuscated other important relationships. In the future, better characterization of the influence of Bifidobacterium in liver and muscle health may allow its use as a pharmaceutical treatment in NAFLD and sarcopenia.

9. Conclusions and future research

9.1. Main findings

In **Chapter 4, "Noninvasive fibrosis tools in NAFLD: validation of APRI, BARD, FIB-4, NAFLD fibrosis score, and Hepamet fibrosis score in a Portuguese population"**, we concluded that the tools examined, APRI, BARD, FIB-4, NFS, and HFS, are accurate in excluding advanced fibrosis in patients with NAFLD. Globally, sensitivity ranged from 46.2% to 90.5% and specificity from 61.6% to 97.7%, with an NPV ≥ 89.9%. The AUROC for each tool were 0.80 for APRI, 0.84 for BARD, 0.88 for FIB-4, 0.88 for HFS, and 0.86 for NFS, which represents excellent discrimination for every tool. The major limitations of this study were its retrospective nature and the ethnic homogeny of the population, while its major strengths were the inclusion of patients from two different centers and the adequate number of patients that exceed the calculated sample size.

In **Chapter 5 "How sarcopenia, muscle mass, muscle strength, and physical performance relate to non-alcoholic fatty liver disease: a systematic review"**, in 53 studies, including 11 unpublished poster abstracts, most found an association between low muscle mass and the presence and/or the severity of NAFLD, and between low muscle strength and presence of NAFLD. The association with low muscle mass, however, was dependent on the definition, as it occurred when muscle mass was index to weight or BMI but not when it was indexed to height. The major limitation of this study was the impossibility of performing a meta-analysis given the vastness of methodology employed. As such, another important conclusion of this study is the need for standardization in this field of research. Other limitations found were the predominance of populations from Asian countries and the repetition of datasets (namely the KNHANES and the NHANES). The major strengths of the study were the incorporation of several databases (PubMed, Web of Science, Scopus, and LILACS) and the inclusion of unpublished poster publications.

In **Chapter 6 "Associations between muscle mass, strength, and performance and non-alcoholic fatty liver disease",** in a cohort study of MetS patients, low muscle mass indexed to weight as found to be associated with the presence of NAFLD, independent of demographic variables and comorbidities but not of other anthropometric parameters such as BMI and waist circumference. As such, we proposed that the interaction between muscle mass, fat mass, and NAFLD needed further

clarification. Regarding NAFLD severity, low muscle mass was associated to significant fibrosis, as defined by NFS > 0.675, in a univariable analysis. The population size did not permit a multivariable analysis of this association, which was one of the major limitations of this work. We found no associations between low muscle strength and poor physical performance and NAFLD. Another limitation of the study was the high prevalence of NAFLD (68.8%), which can be explained by the selection of patients from a MetS population, but which may pose a statistical obstacle for association recognition. The major strength of this study was the clear definition of sarcopenia and the inclusion of all its parameters; moreover, the tools used for assessment of muscle mass, strength, and performance followed the recommendations in most recent guidelines on the issue.

In **Chapter 7 "Fibroblast growth factor 21 and myostatin are higher in females with NAFLD and correlate with dysmetabolism and lower muscle mass, strength, and performance"**, we found specific sex differences regarding the metabolic disturbances, body composition, and the pattern of myokines expressed in patients with NAFLD. In females, patients with NAFLD had higher FGF21 and myostatin; they also had worse metabolic parameters (higher HbA1c, HOMA-IR, and TG, and lower HDL-c), lower muscle mass (as SMI), lower muscle strength (as chair stand time), and worse physical performance (as gait speed and as SPPB). Correlating myokines with these other variables, in females, FGF21 was positively correlated with HbA1c, HOMA-IR, and TG, and negatively correlated with HDL-c, muscle mass, and physical performance. Myostatin was negatively correlated with muscle mass, muscle strength, and physical performance. As such, our findings suggested a possible role of FGF21 in the association of dysmetabolism and NAFLD, and of myostatin in sarcopenia and NAFLD. Our major limitations in this study were the high prevalence of NAFLD and of dysmetabolism (as this was a population of patients with MetS), and the fact that the population very ethnically homogenous and from a single hospital center.

In **Chapter 8** "**Gut microbiome composition and its associations with NAFLD and low muscle mass**", no differences were detected in gut microbiome composition between patients with and patients without NAFLD. However, in patients with NAFLD, those that presented low muscle mass had less relative abundance of *Bifidobacterium*, while when looking at patients with normal SMI, patient with NAFLD had a tendency to

lower abundance of *Bifidobacterium*, suggesting a role of this genus in both NAFLD and muscle mass. Our major limitation was the small sample size with a low number of patients with low muscle mass and with low physical performance, that prevented analyses regarding these variables, which would have been of particular interest since the *Bifidobacterium* has been positively associated with muscle mass.

9.2. Future research

Several questions remain to be answered regarding the topics of this Thesis.

The association of low muscle mass and NAFLD that was described in **Chapter 6 "Associations between muscle mass, strength, and performance and non-alcoholic fatty liver disease"** should be replicated in other settings, particularly in primary care and in health surveys; since our patients were selected from a tertiary hospital center, one of our main limitations was the likely inclusion of patients with more advanced disease. The findings in this chapter also warrant further characterization, which could be achieved via a prospective study, to determine causative links between these variables.

An association between muscle parameters and NAFLD severity could not be analyzed in our cohort population (**Chapter 6 "Associations between muscle mass, strength, and performance and non-alcoholic fatty liver disease")** given the small percentage of patients with advanced fibrosis. Increasing the sample size could, therefore, afford more clarification. The use of VCTE would also allow for more easy analyses since its results can be viewed as a continuous as well as a categorical variable.

The sex differences detected in **Chapter 7 "Fibroblast growth factor 21 and myostatin are higher in females with NAFLD and correlate with dysmetabolism and lower muscle mass, strength, and performance"** require further explanation. It would be interesting to analyze the association between the variations here described and sexual hormones, like testosterone and estrogen. The inclusion of premenopausal women could add insight on the role of these hormones.

The importance of the gut microbiome in muscle health approached in **Chapter 8** "**Gut microbiome composition and its associations with NAFLD and low muscle**

mass" would be served by further studies. Associations with muscle strength and physical performance need characterization with an augmented sample size. It could also be of clinical significance an interventional study analyzing the impact of a *Bifidobacterium* probiotic in patients with low muscle mass. Also, the impact on dietary patterns and their changes on the gut microbiome of patients with NAFLD and with low muscle mass could be analyzed.

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