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Università Campus Bio-Medico di Roma

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Innovative measures of frailty in patients with bone impairment

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STETEMENT OF ORIGINALITY

The works reported in this thesis were performed at the University Campus Bio-Medico, Rome, Italy.

The author designed the trials included in this thesis and analysed, described and discussed the results.

I hereby state that this thesis entitled **"Innovative measures of frailty in patients with bone impairment"**

has not been submitted for a degree or other qualification at any others universities.

Rossella Del Toro

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ABSTRACT

Frailty is a condition characterised by a declining function across several homeostatic systems leading to increased vulnerability to stressors and the risk of adverse health outcomes. Today frailty is an emerging global health burden with major implications for clinical practice and public health.

Both in literature and in clinical practice standard measurements of frailty have not been unanimously agreed upon yet. Many reviews highlighted the need for standard instruments to diagnose frailty, as these would allow the heterogeneity of frailty to reduce worldwide.

Scientists are asked to expand the knowledge of pathophysiology and of the natural course of frailty, identifying precise biomarkers and evaluating changes in organs.

The overall aim of my PhD was to find out the biological changes that may lead to frailty and vulnerability, thus understanding the relation between the changes in different organs and in tissues.

This thesis includes three research projects: the first one focused on the effective identification of biomarkers involved in the bone-vascular axis, which might significantly improve the prediction of atherosclerotic cardiovascular disease; the second research project investigated the prevalence of vertebral fractures in a population with a very high cardiovascular risk and it evaluated a possible relationship with frailty and daily calcium intake; the third one focused on elderly people affected by COVID pneumonia, investigating the behaviour of skeletal muscle mass, thoracic aortic calcifications and bone mineral density through measures obtained from CT scans of the chest.

1st Research project: this study was based on much pre-clinical and clinical evidence suggesting an interplay between atherosclerosis and osteoporosis, both chronic diseases involved in ageing and frailty. Bone turnover biomarkers were studied for a possible role in the bone-vascular axis, thus they were also considered as novel markers of vascular health. The aim was to evaluate the association between serum concentrations of soluble bone biomarkers (Osteopontin, Osteoprotegerin, Klotho and Sclerostin) and atherosclerotic disease severity assessed through coronary angiography, gold standard for the diagnosis of coronary artery disease and carotid

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doppler ultrasound. We proceeded by testing the associations of these candidate bone biomarkers with traditional cardiovascular risk factors. The results of this phase demonstrated that elevated serum levels of OPG are independently associated with advanced atherosclerosis, confirming a link between bone metabolism and vascular disease.

2nd Research project: the second part of the project looked at the presence of vertebral fractures on spine radiographs, which are often neglected in clinical practice. In addition, this study highlighted elderly people's nutritional aspects, in particular the intake of calcium as calcium is one of the elements involved both in the development of osteoporosis and in the increase of atherosclerosis. The aim was to investigate the prevalence of vertebral fractures in a population with very high cardiovascular risk, and to evaluate a possible connection to frailty and the daily intake of calcium from food. The results of this investigation demonstrated how unrecognised vertebral fractures and a low daily calcium intake are associated with a higher frailty score, confirming the link between bone health and vascular diseases.

3rd Research project: this study was influenced by the recent COVID pandemic, which had the most dramatic impact on frail older people. Thoracic CT, currently used to evaluate lung extension and the severity of pneumonia, could be used to investigate other aspects, not only as a marker of frailty in elderly people, but also as a predictor of poor prognosis in COVID pneumonia. The hypothesis was that the complex pathophysiology of frailty in elderly people involves common pathways to muscle mass, macro vascular calcifications and bone density.

The results of this research demonstrated how vascular calcification is inversely related to bone mineral density and muscle mass density, while bone and muscle density are directly correlated. Finally descending thoracic aorta calcium score has a good diagnostic power to identify an increased risk of death in older adults with SARS CoV 2 pneumonia.

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ABBREVIATIONS

FI: Frailty Index

GH: Growth Hormone

IGF-1: Insuline-like Growth Factor-1

DHEA-S: Dehydroepiandrosterone Sulfate

IL-6: Interleukin 6

PCR: C-reactive protein

WBC: White blood cell

ADL: Activities daily living

ICFSR: International Conference of Frailty and Sarcopenia Research

CFS: Clinical Frailty Scale

EFS: Edmonton Frailty Scale

FI-CD: Frailty Index of Accumulative Deficits

CGA: Comprensive Geriatic Assessment

FI-CGA: Frailty Index can be derived from CGA

BMD: Bone Mineral Density

CV: Cardiovascular

LDL: Low-density lipoproteins

GBD: Global Burden of Disease

ACS: Acute coronary syndrome

AMI: Acute myocardical infraction

CKD: Chronic kidney disease

DM: Diabetes mellitus

GFR: Glomerular filtration rate

PAD: Peripheral artery disease

SCORE: Systematic coronary risk estimation

TIA: Transient ischemic attack

ABI: Ankle Brachial Index

VSMCs: Vascular smooth muscle cells VSMCs

BMP2: Bone morphogenetic protein 2

oxLDL: oxidizing LDL

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MGP: matrix Gla protein

VSMC: vascular smooth muscle cells

OPG: Osteoprotegerin

SOST: Sclerostin

OPN: Osteopontin

BMP: Bone morphogenetic proteins

KL: Klotho

PTH: Parathyroid Hormone

RAAS: Renin-Angiotensin-Aldosterone-System

cIMT: carotid intima-media thickness

eGFR: estimated glomerular filtration rate

ELISA: Enzyme-linked immunosorbent assay

SD: Standard deviation

ASA: Acetylsalicylic acid

ARBs: Angiotensin receptor blockers

FGF: Fibroblast growth factors

SENECA: Survey in Europe on Nutrition and the Elderly

SQ: semi-quantitative

VRD: vitamin receptor D

LARN: Reference Levels of Nutrients and energy intake for the Italian population

AMI: acute myocardial infarction

WHO: World Health Organization

IDI-ICRSS: Immaculate's Dermatopathic Institute

VES: erythrocyte sedimentation rate

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

CT: computed tomography

CAC: Coronary artery calcification

TAC: Thoracic aorta calcifications

HU: Hounsfield units

EWGSOP-2: European Working Group On Sarcopenia in Older People 2

SMM: Skeletal muscle mass

DTAC: Descending thoracic aorta calcification

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ROI: Region of interest

GE: General Electric

INR: International Normalized Ratio

ALT: Alanine amino transferase

LDH: Lactic dehydrogenase

OSO: Osteosarcopenic obesity

AUC: area under the curve

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CHAPTER 1:

GENERAL BACKGROUND ON FRAILTY

1.1 Introduction

Frailty is a clinical condition characterized by a reduction in physiological capacity across several organ systems causing increased susceptibility to stressors. It is associated with increased mortality, hospitalization, falls and admission to long-term care (1, 2).

The condition of frailty is an emerging global health burden with major implications for clinical practice and public health (3).

Frailty occurs in adults at any age, but it is more prevalent in older adults. For this reason, the global impact of frailty is expected to increase due to population ageing (3).

The concept of frailty has drawn increasing interest during the last 30 years. Although a large part of the literature has highlighted the importance of this topic, the correct criteria through which to identify frailty have not been defined yet (4-6).

The two main paradigms defining frailty are:

• the biomedical paradigm, which defines frailty as a physiological syndrome that is characterized by the reduction of functional reserves and a reduced resistance against stressors; these reductions are themselves caused by a collective decline of multiple physiological systems causing vulnerability and adverse consequences (7);

• the bio-psycho-social paradigm, which defines frailty as a dynamic state affecting an individual who shows deficits in one or more functional domains (physical, psychic, social). These deficits are caused by different variables increasing the risk of adverse results in terms of health (8).

Rockwood proposed an alternative definition of frailty with the Frailty Index (FI): a comprehensive list including a number of deficits collected over a period (9). This definition is based on the idea that frailty is caused by the disorganization of physiological systems, and that this aspect can be evaluated by assessing functional status, diseases, physical and cognitive deficits, psycho-social risk factors and geriatric

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syndromes; in this case the objective is to envision as accurately as possible the risk of adverse events.

Over the past years the concept of frailty has also had the advantage of shifting the approach to elderly patients which is usually focused on the disease or the organ towards a multidimensional approach to health in its various aspects (9).

Nowadays the concept of frailty is increasingly being used in primary, acute, and specialist care. Nevertheless, the effective conversion from research to clinical remains a challenge in the present and future.

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1.2 Epidemiology

The prevalence of frailty in the world is not known for two main reasons: the first reason is that research about frailty was mostly conducted in developed countries; the second is that the various studies used different working definitions of frailty (3).

Recently 22 European countries were involved in the Joint Action ADVANTAGE, a systematic review and meta-analysis of literature on the prevalence of frailty in these countries. One of the main aims of the European project ADVANTAGE was to collect and review critically the current literature on subjects concerning the management of frailty. The meta-analysis included sixty-two papers which estimated an overall prevalence of frailty of the 18% in adults aged 65 years and over (10). However, this review also demonstrated that the results were not homogeneous, as the population group enrolled in the studies was heterogeneous and the tools that were used to define frailty varied (10). Other similar meta-analyses of the rates of frailty prevalence in low and middle-income countries revealed the same problem with the heterogeneity of groups and tools (11).

Furthermore, systematic reviews analyzed the frailty prevalence in specific clinical settings: they calculated a prevalence of 5-29% in subjects with HIV infection (12); 37% in patients with end-stage renal disease (13); 42% in patients with solid or hematological malignancies (14). Finally, the prevalence of frailty in long-term care is 53% and it is one of the highest rates (15).

Despite the difficulty in identifying a specific prevalence, several studies have showed common patterns. The prevalence of frailty is higher in low socio-economic strata and in ethnic minorities, moreover such prevalence increases with older age and it is higher in women than in men (16, 17).

In older adults frailty becomes an important risk factor for mortality (18). Many studies confirmed the association between frailty and mortality in different kind of settings and populations. Frailty is commonly known to increase the risk of falls, fractures, disability, hospitalization, and to be associated with a lower quality of life, loneliness, depression, cognitive impairment, dementia and admission to nursing homes (3).

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1.3 Pathogenesis

There is increasing evidence that the onset of frailty is due to a dysregulation of stress response systems, including the immune, endocrine and energy response systems. Different factors contribute to the aetiopathogenesis of frailty: ageing-related molecular changes, genetics, environmental exposure and inflammatory diseases. These modifications determine an increase of inflammatory cytokines and hormonal dysregulation, resulting in loss of skeletal muscle and muscle strength, which is also identified as sarcopenia (19).

During ageing, there are several hormonal changes that have been associated with frailty. In particular, the increase of cortisol levels and the decrease of GH, IGF-1, DHEA-S, sex steroids and 25(OH) vitamin D (20) have been observed. The modifications described above contribute to muscle decline and to the activation of inflammatory pathways.

There is a link between inflammatory cytokines and the pathophysiological changes of tissues that are related to frailty. In community-dwelling frail older adults, serum levels of interleukin 6 (IL-6), C-reactive protein (PCR), white blood cell (WBC) and monocyte count increase (21-25). Moreover, the association between frailty and coagulation is proved, indeed the activation of immune system may start the clotting cascade (25). At the same time, immune defenses struggle to protect frail older people from pathogens, which makes them more vulnerable to disease and cancer and less responsive to vaccines (26).

Other mechanisms involved in the pathogenesis of frailty are: impairment of glucose metabolism (27), alteration of the autonomic nervous system (28), dysregulation of renin-angiotensin system and of mitochondria. Physical frailty takes several different shapes when untreated and its initial heterogeneous manifestations often lead to different trajectories of frailty progression (29). Nevertheless, two of the epidemiological studies about frailty, namely the Longitudinal Ageing Study Amsterdam with 15-year follow-up and the InCHIANTI study with 9-year follow-up, reported equal results with regard to the initial aspect of frailty. The first physical component of frailty to manifest itself is often exhaustion, followed by slowness of gait, lowered physical activity and weakness (30); while weight loss seems to develop later than other physical aspects of frailty (30, 31).

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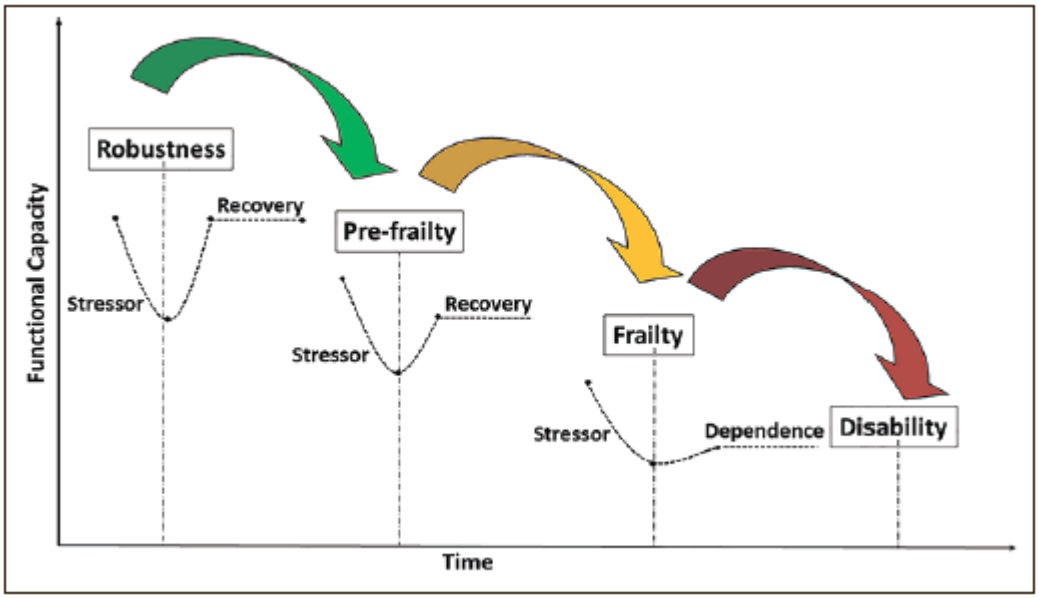
The rate of functional decline in frailty, especially during the last year before death, is very slow compared with other diseases. The functional decline associated with frailty is gradual, continuous, and it begins several years before death (32).

In the twenty-first century the challenge is the early identification of frailty during its functional decline, as well as the management of frailty with targeted interventions able to stop, slow or reverse the cascade decline (Fig. 1).

FIG. 1 *Cascade of functional decline in older adults without any interventions (33).*



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1.4 Phenotype

The definition of frailty phenotype as a geriatric syndrome was suggested and tested by Linda P. Fried in the Cardiovascular Health Study, a cohort study of over 5,300 community-dwelling elderly people conducted in the US. Frailty is a condition based on the presence of specific phenotypic criteria: weakness, as measured by low grip strength, slowness, as measured by slowed walking speed, low level of physical activity, asthenia or subjective exhaustion and involuntary weight loss. None of the above five criteria are classified as non-frail, one or two criteria identify a pre-frail stage while the presence of three or more of the five criteria indicates frailty phenotype (1, 34).

Two of the most important distinctions are between frailty and disability, which is measured by impairment in activities daily living (ADL), and between frailty and comorbidity, which is characterised by two or more diseases. Certainly there could be overlaps between these three distinguished conditions, even if they are distinct clinical entities (Fig. 2). Many frail people are disabled, while not all disabled persons are frail. Moreover, the sole presence of two or more clinical diagnoses may not identify a frail older adult, yet when the multiple diseases worsen, or when they are not adequately treated, and/or when more comorbid conditions accumulate, these patients may develop frailty. Nevertheless, disability and comorbidity are important confounding factors that deserve careful consideration in frailty assessment (35).

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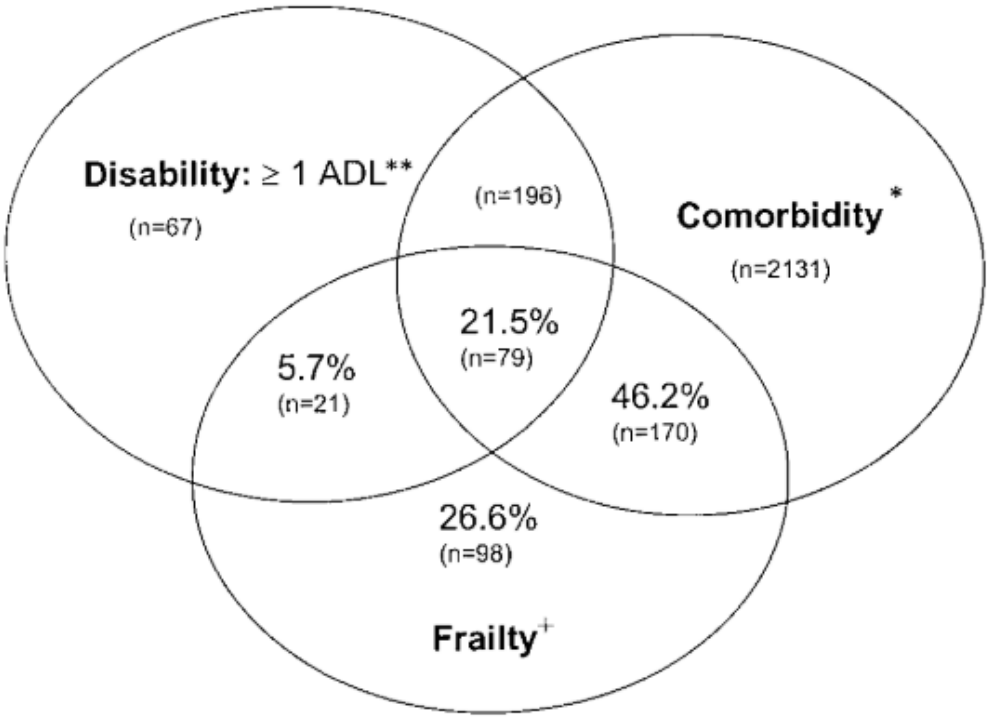
FIG. 2 *Venn diagram displaying the extent of the overlap of frailty with ADL disability and comorbidity (1). *Comorbidity: ≥ 2 diseases: myocardial infarction, angina, congestive heart failure, claudication, arthritis, cancer, diabetes, hypertension, COPD. **Disabled: ADL disability.*



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1.5 Assessment

Nowadays, there is no unanimous identification in literature and in clinical practice of the standard measurements of frailty. Many reviews evidenced the need for standard instruments to diagnose frailty, as these would allow the heterogeneity of frailty to reduce worldwide (36).

The international Conference of Frailty and Sarcopenia Research (ICFSR) strongly recommends opportunistic screening for frailty in all adults over 65; the screening tools should be validated and suitable to the specific setting or context (33). Currently there exists a great number of frailty measurements and the debate about which tests are more suitable and efficient is ongoing.

The most widely used screening tests are the Rockwood's Clinical Frailty Scale (CFS) (37), the International Association of Nutrition and Ageing (IANA)'s FRAIL scale (38) and the Edmonton Frailty Scale (EFS) (39).

The CFS is a well validated frailty measurement, based on clinical judgment on a nine-point pictorial scale from 1 (very fit) to 9 (terminal ill) (37). A score ≥ 5 is diagnostic of frailty. It was validated as an adverse outcome predictor in hospitalized older people (37).

FRAIL consists of five criteria: Fatigue, Resistance, Ambulation, Illness and Loss of weight (38). An older adult is defined as frail through the presence of three or more of the above components. FRAIL has been found to be predictive of mortality in specific populations (38).

EFS contains nine components: cognition, general health status, self-reported health, functional independence, social support, polypharmacy, mood, continence and functional performance (39). It is a valid instrument for the identification of frailty in hospital setting (39). It is scored out of 17: not frail (0-5), apparently vulnerable (6-7), mildly frail (8-9), moderately frail (10-11) and severely frail (12-17).

The researchers agreed to perform a clinical assessment in all older adults who resulted frail or pre-frail to the screening (33).

In 2001 Fried proposed a frailty phenotype measurement, which assesses five physical components: unintentional weight loss, weakness, exhaustion, slowness and low physical activity (1). Pre-frail is classified when one or two components are present, while frail requires three or more criteria. This measurement was used in

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multiple epidemiological studies and it demonstrated to be predictive of adverse clinical outcomes, including mortality (1). Fried's Frailty Phenotype does not include the psychosocial components of frailty.

Rockwood and Mitnitski, also in 2001, realised their accumulated deficits model of frailty (40). The idea is that a higher frailty is the expression of the increasing number of comorbidities, symptoms, diseases, disabilities or any other deficiency (40). The Frailty Index of Accumulative Deficits (FI-CD) is expressed as a ratio, it is validated and it has been applied to multiple dataset including the Survey of Health and Ageing and Retirement (SHARE) study in Europe (41, 42). Both in hospital and community setting, FI-CD has a higher predictive value of adverse clinical events and outcomes (43, 44).

Before the introduction of the concept of clinical frailty the Comprensive Geriatric Assessment (CGA) was designed to identify disability in older adults (5). It is the global classical assessment for older people and it includes medical, nutritional, functional and psychological aspects by a multidimensional team (5). Frailty Index can be derived from CGA (FI-CGA): at the beginning it was based on ten-domain index with 14 CGA components (45, 46), then Rockwood expanded the test including 52 CGA components (47). FI-CGA has been found to predict outcomes in multiple fields and settings.

In general two methodological points of view are used to study frailty and they reflect two different conceptual interpretations: the first, theorised by Fried, sees frailty as pathophysiological geriatric syndrome and it is based on the identification of biological-functional characteristics that define a specific phenotype (7); the second predicts frailty as a condition due to the progressive accumulation of clinical and functional deficits, and it uses a frailty index which quantitatively expresses the number of deficit of the single individual (48).

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1.6 Management

Care plans for frailty have not been widely developed or specifically identified yet. Despite this situation, there is general consensus on the need to implement strategies that can mitigate the daily impact of frailty on the quality of life and overall health status.

Such care plans should include the treatment of polypharmacy, sarcopenia, weight loss or undernutrition and exhaustion (depression, anemia, hypotension, hypothyroidism and vitamin B12 deficiency) (33). Until now a low certainty of evidence has been gathered concerning the effectiveness of individually tailored care plans for older adults with frailty (33).

Recent ICFSR guidelines for sarcopenia discuss specific interventions to improve muscle strength, function and muscle mass. A multi-component physical activity program should be offered to older people with frailty as well as to people with pre-frailty as a preventative strategy (33). A systematic review analyzed 21 randomized trials with a total of 5275 older adults and 33 exercise interventions aimed at preventing frailty (49). The results showed that group physical programs were more effective in improving frailty than individual sessions, measured through any validated scale or index (49). In 2018 a randomized trial of 1637 French community-dwelling older adults showed that the intervention group had a reduction of both numbers of comorbidities and persistent deficits compared to usual care (50). The intervention included lifestyle changes: cognitive training, nutrition counselling and advice on physical activity, factors which demonstrated an advantageous result on a variety of frailty components (50). The benefits of physical activity are the following: increased mobility, better performance of the activities of daily living (ADLs), improved gait, decreased number of falls, improved bone mineral density (BMD) and enhancement of general wellbeing. Even the frailest older adults are likely to benefit from exercise at almost any level that can be safely tolerated (20).

In cases where weight loss or undernutrition have been diagnosed, protein/caloric supplementation is among the most promising interventions in the prevention and reduction of frailty (33). However, considering intervention trials little evidence exists to support protein/caloric supplementation for frail people. On the other hand, dietary food quality seems to influence the progression of frailty condition (51). A meta-

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analysis published in 2018 described how a good adherence to a Mediterranean diet diminishes significantly incident frailty (52). Furthermore the lack of micronutrients like folate, Beta-carotene, vitamin A, C and E is also associated with the progression of frailty (53). Overall, in patients with weight loss as a frailty component attention should be focused on the treatable causes identified by the MEELS-ON-WHEELS mnemonic (Fig. 3) (54, 55).

FIG. 3 *MEELS-ON-WHEELS mnemonic (33).*

Medications
Emotional (depression)
Alcoholism, anorexia tardive, abuse (elder)
Late life paranoia
Swallowing problems

Oral problems
Nosocomial infections, no money (poverty)

Wandering/dementia
Hyperthyroidism, hypercalcemia, hypoadrenalism
Enteric problems (malabsorption)
Eating problems (eg, tremor)
Low salt, low cholesterol diet
Shopping and meal preparation problems, stones (cholecystitis)

The effects of a pharmacological approach in the treatment of the frailty syndrome have not been adequately evaluated. Although the pathogenesis of frailty is based on the activation of inflammatory pathways and the dysregulation of endocrine systems, no hormone or antinflammatory therapy has showed to have significant benefits for frailty (33).

Several other meta-analyses of randomized trials found a reduction in falls thanks to vitamin D supplementation (56-60). Nowadays new knowledge about the extra skeletal effects of vitamin D confirms its important role to preserve the integrity of both muscle and nervous system tissue, as well as its importance as immunomodulating molecule (61). The daily intake of vitamin D recommended in older adults should be at least 700 to 1000 international units. Considering the favorable pharmacological and safety profiles of vitamin D, further studies aiming at investigating the clinical utility in the prevention and treatment of frailty are indicated.

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Finally, another important area of intervention is the prevention of biological, socioeconomic and environmental stressors; it becomes clear that multimodal strategies of intervention should be considered for the frail elderly (33).

Finally, another important area of interventions is to prevent biological, socioeconomic and environmental stressors, in this way a multimodality strategies intervening should be considered for the frail elderly (33).

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CHAPTER 2

RESEARCH PROJECT 1: *Association of bone biomarkers with advanced atherosclerotic disease in people with overweight/obesity*

2.1 ROLE OF THE BONE-VASCULAR AXIS

2.1.1 Background

Both the increase in average age and the consequent increase in elderly population are associated with the development of osteoporosis and vascular diseases. In this area, an increasing number of biological and epidemiological evidence supports a link between cardiovascular disease and bone biology. Several cross-sectional and longitudinal studies found an inverse relationship between BMD and vascular calcifications (62, 63). Furthermore, prospective studies have associated a low BMD with the increased risk of cardiovascular (CV) events (62). This developing evidence raises an interesting question about the existence of a possible pathophysiological mechanism that can lead to both bone impairment and vascular disease and/or vascular calcifications. Overall, the existence of a bone-vascular axis is strongly suggested, and the search for bone metabolism markers related to CV disease currently represents an open field of research. Cardiovascular risk stratification is essential for individualised treatment and CV prevention strategies. Novel CV biomarkers may significantly improve the prognostic value of available risk scores based on classical risk factors. Importantly diabetes has been shown to increase bone fragility by interfering with different bone metabolic pathways; and the presence of vascular complications further increases the risk of bone fractures (64).

This further highlights the relevance of searching for novel biomarkers of CV disease related to bone metabolism.

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2.1.2 Atherosclerosis

It is well known that atherosclerosis is a pathological process caused by the formation of focal plaques (made of fibrous tissue and lipid material) which affect the intimate and medium tunics of medium and large arteries. It is a multifocal and progressive systemic disease that manifests itself later than its onset (65). The protagonist of the latest theories on the pathogenesis of atherosclerosis is the endothelial dysfunction, which is caused by different insults. Chronic damage to the endothelium promotes the loss of its vascular homeostasis, thus allowing the adhesion of platelets and monocytes. Consequently, vascular permeability is compromised, favouring the penetration of plasma substances including low-density lipoproteins (LDL) and inflammation cells. These processes are amplified by the on-site production of vasoactive molecules, cytokines and growth factors which determine the progression of local damage, resulting in the progressive thickening of the arterial wall up to the formation of the atheromatous plaque (65, 66).

Today atherosclerosis is the main cause of mortality in industrialized countries. It is also one of the most important causes of morbidity, disability and hospitalization in the world. In 2016 the third Global Burden of Disease Study (GBD) – the largest and most comprehensive observational study on global health – published the following results (67). In 2015 32.1% of global deaths were due to cardiovascular disease, confirming the 1980 ranking that reported cardiovascular disease as the leading cause of death. Nevertheless, the age-adjusted mortality rate due to cardiovascular disease decreased about 15.6% since 1980. In Italy the situation is in line with the world ranking, in fact cardiovascular diseases were the first cause of death, although showing a reduction of deaths from cardiovascular causes of 10.84% out of total deaths (67). These results were due to the implementation of health interventions which aimed at preventing and treating these pathologies more effectively, while generating a chronicity of the disease.

Atherosclerosis is present in all individuals over 20 years of age; only pathological complications due to the presence of atherosclerotic lesions establish the disease (68). It is difficult to assess the atherosclerosis degree of each patient and assessment may require invasive investigations. Thus it is more effective to classify patients on the

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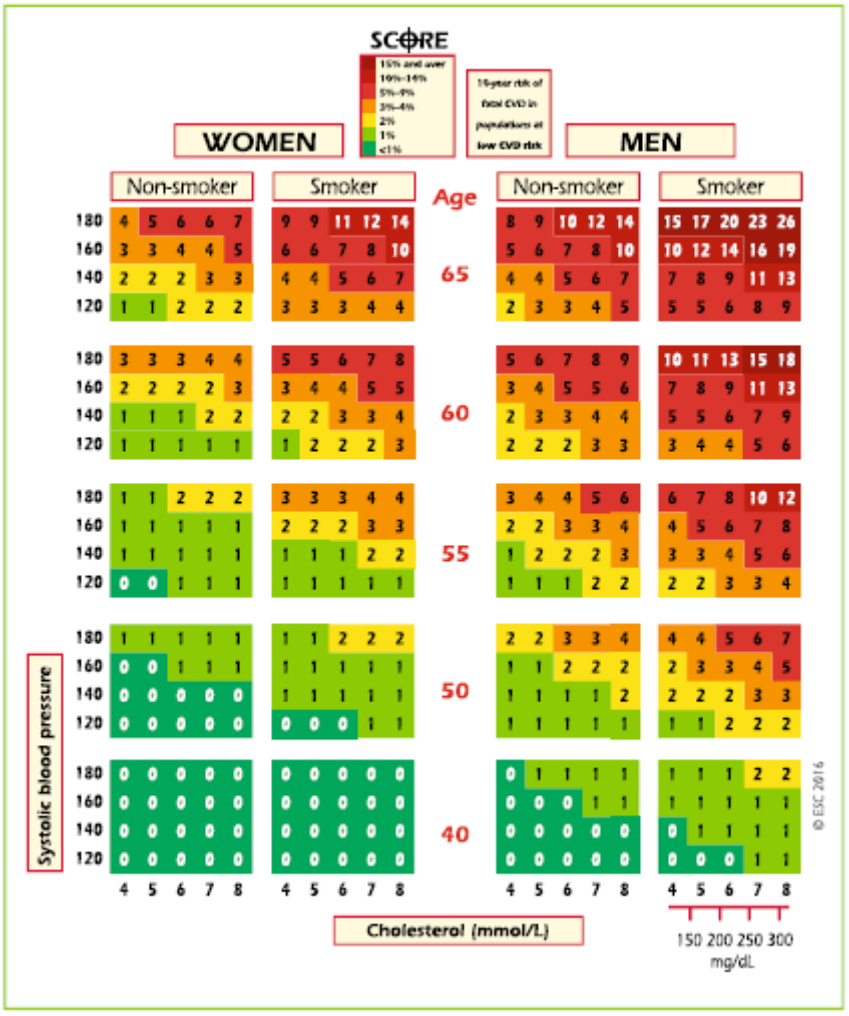
basis of the statistical risk of complications, specifically myocardial infarction (Fig. 4) and stroke.

FIG. 4 *Systematic Coronary Risk Estimation (68). 10-year risk of fatal cardiovascular disease in populations of countries at low cardiovascular risk (including Italy) based on the following risk factors: age, sex, smoking, systolic blood pressure, total cholesterol. CVD: cardiovascular disease*



According to numerous epidemiological and intervention studies, the risk of atherosclerotic events is related to the presence of pathophysiological and/or behavioral parameters (sex, age, blood pressure, glycaemia, cholesterol and smoking). More specifically, several scores have been formulated for the stratification of cardiovascular risk (Fig. 4) and for the identification of the risk category of each subject (Fig. 5).

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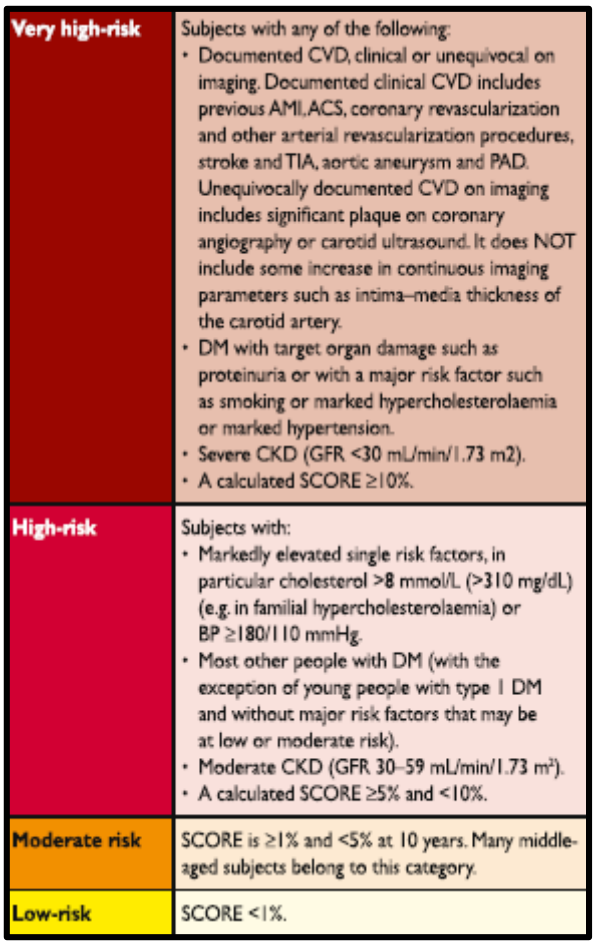
FIG. 5 *Risk categories (68). ACS: acute coronary syndrome; AMI: acute myocardial infarction; BP: blood pressure; CKD: chronic kidney disease; DM: diabetes mellitus; GFR: glomerular filtration rate; PAD: peripheral artery disease; SCORE: systematic coronary risk estimation; TIA: transient ischemic attack.*



Very high-risk	Subjects with any of the following: • Documented CVD, clinical or unequivocal on imaging. Documented clinical CVD includes previous AMI, ACS, coronary revascularization and other arterial revascularization procedures, stroke and TIA, aortic aneurysm and PAD. Unequivocally documented CVD on imaging includes significant plaque on coronary angiography or carotid ultrasound. It does NOT include some increase in continuous imaging parameters such as intima–media thickness of the carotid artery. • DM with target organ damage such as proteinuria or with a major risk factor such as smoking or marked hypercholesterolaemia or marked hypertension. • Severe CKD (GFR <30 mL/min/1.73 m2). • A calculated SCORE ≥10%.
High-risk	Subjects with: • Markedly elevated single risk factors, in particular cholesterol >8 mmol/L (>310 mg/dL) (e.g. in familial hypercholesterolaemia) or BP ≥180/110 mmHg. • Most other people with DM (with the exception of young people with type 1 DM and without major risk factors that may be at low or moderate risk). • Moderate CKD (GFR 30–59 mL/min/1.73 m²). • A calculated SCORE ≥5% and <10%.
Moderate risk	SCORE is ≥1% and <5% at 10 years. Many middle-aged subjects belong to this category.
Low-risk	SCORE <1%.

If necessary after the cardiovascular risk assessment more imaging techniques are used: these can improve risk prediction and more importantly they can guide the decision-making and therapeutic process. The main techniques that are used are the following: carotid echocolordoppler, echocardiogram, Ankle Brachial Index (ABI) and any chosen coronary angiography (68). Therapy consists of modifying risk factors: lifestyle changes thanks to a balanced diet, low in saturated fat, increased physical activity, quit smoking, glucose control and blood pressure control with a low-sodium diet or with antihypertensive therapy if necessary. Last but not least, the reduction of low-density cholesterol and triglycerides through a lipid-lowering diet for subjects with low risk of developing CV diseases, and reduction through the use of lipid-lowering drugs for medium and high-risk individuals (68).

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2.1.3 Arterial calcification

The distinctive feature of atherosclerosis is the accumulation of foam cells – also called lipid-laden macrophages – in the sub-endothelial area of the arterial wall. These cells contain cholesterol and promote inflammatory pathways in the arterial walls, leading to multiple fatal pathological consequences such as hemorrhage, rupture and calcification (69-71).

Up until the first half of the twentieth century vascular calcification was dismissed as a passive process caused by cellular death. More recent research has instead revealed how vascular calcification is an active process of extracellular matrix mineralization (72).

In the earliest pathological description of atherosclerosis Rudolph Virchow recognized osteo-fibrogenic differentiation as one of the factors involved in the biology of vascular calcium accumulation (73). Not surprisingly molecular markers of osteogenic activity can be found in all mineralised arterial segments.

According to clinical and pathology criteria there are three types of arterial calcification (Fig. 6).

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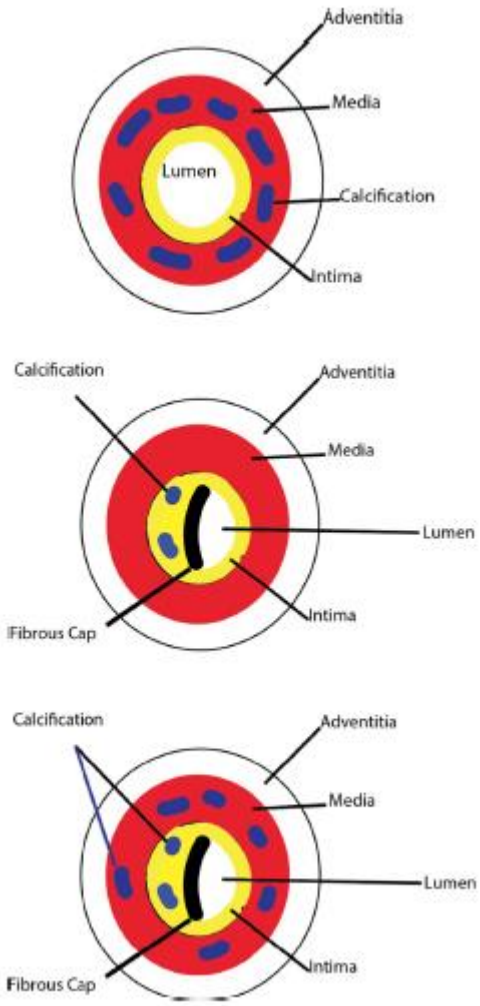
FIG. 6 *Types of vascular calcifications (74)*



Medial Arterial Calcification (Monckeberg's arteriosclerosis)

Intimal Atherosclerosis

Arterial Calcification of Chronic Kidney Disease

Intima calcification is the most common form of calcification and it is mostly caused by dyslipidemia and inflammatory factors such as lipoproteins and cytokines; arterial medial calcification (also known as calcific sclerosis of the Monckeberg media) is related to age, diabetes mellitus and chronic renal failure. Individuals affected by medial calcification have a higher risk of mortality from cardiovascular events and an increased risk of amputation (75, 76).

Medial arterial calcifications are concentric processes of the tunica media and they are associated with biomineralisation and fibrosis of the vessel wall.

Intimal atherosclerosis is a prototypic lesion constituted as an eccentric, lumen deforming, remodeling lesion, and it is characterized by fibrous-cap, foam cells, lipoprotein deposits, focal infiltration of inflammatory cells and elastinolysis. In this kind of lesion there are also mineralizing matrix vesicles produced by vascular smooth

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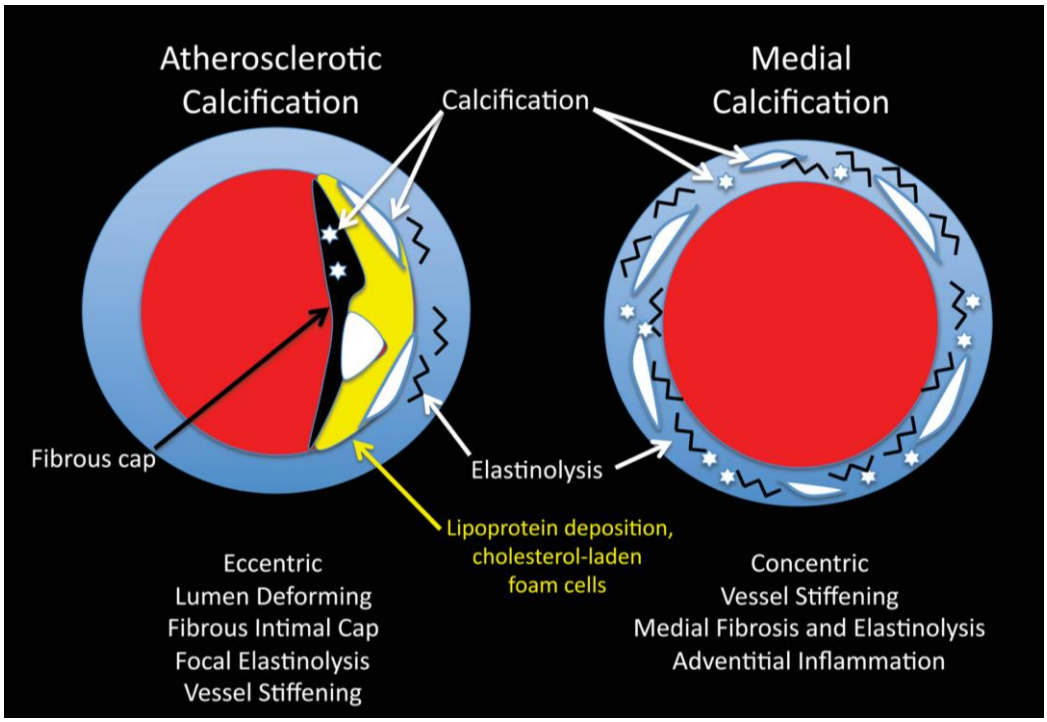
muscle cells (VSMCs) and dotty fibrous cap calcifications, which are precursors to the ruptured plaque (77) (Fig. 7).

FIG. 7 *Atherosclerotic calcification and medial calcification (77)*



Out of the many studies examining the pathology of vascular calcifications few focused on heterotrophic bone formation in the vessel. In 1998 Woolley et al. examined the late stage of atherosclerotic plaques in the carotid arteries wall: the study found that a percentage of the plaques contained mineralized bone, showing features also found in skeletal osteogenesis, such as osteoid, mineralized trabeculae, osteocytes, multinucleated osteoclasts, mast cells and macrophages (78). In 2002 Hunt et al. examined plaques which had been removed following carotid endarterectomy in patients with stenosing disease and demonstrated the presence of mature lamellar bone in plaques in 13% of the patients (79). Within the subject area of the ossification of the middle tunic, in 2008 Soor et al., analysed arterial wall segments of patients undergoing lower limb amputation, revealing the presence of calcification of the media and atherosclerotic plaques in 76.2% of the arterial segments which were removed (80).

One of the first bone markers to be identified in the artery walls was the alkaline phosphatase, which was known for its involvement in bone mineralization processes

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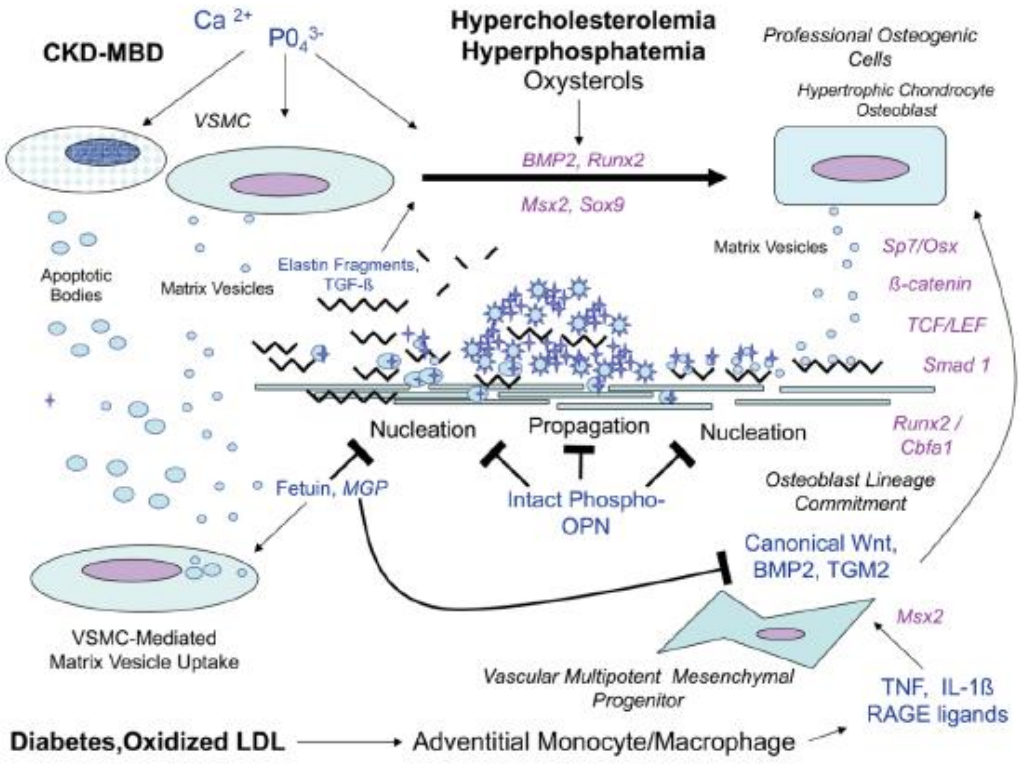
(81). The bone morphogenetic protein 2 (BMP2) was expressed in the calcifications of human atherosclerotic plaques (82). Subsequently oxidized LDL (oxLDL) and hyperglycemia were recognized as potent inducers of endothelial BMP2, being correlated with the vascular ossification grade (83, 84). Confirming these data in atherosclerotic mice, BMP2 inhibition by part of the matrix Gla protein (MGP) resulted in a reduction in the number and size of vascular calcifications (85).

Despite this evidence, the relation between the progression of atherosclerosis and arterial medial calcification has not been specified yet. Vascular mineralization is regulated by complex overlapping processes yet ones that are distinct from those that control skeletal bone formation. Metabolic and inflammatory insults play a key role in inducing the osteogenic differentiation of vascular mesenchymal cells, both in intima and in medial calcifications (Fig. 8).

FIG. 8 *Vascular osteogenic cell origins, functions and phenotypes in arterial calcification (77)*



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2.1.4 Osteokins involved in atherosclerotic disease

Vascular calcification is a complex process whose mechanisms are similar to those of bone formation; one example of such mechanisms is the mineralization of vascular smooth muscle cells (VSMC). In fact, such VSMC differentiates into osteoblastic-like cells following exposure to various stresses. As discussed previously, many osteo-metabolic markers are expressed within the atherosclerotic plaques.

The factors involved in the pathogenesis of bone impairment and vascular calcification are numerous and they include proteins, hormones, lipids and vitamins as summarized in Table 1.

TAB. 1 *Molecules involved in bone metabolism and in the pathogenesis of vascular calcifications (86)*

MOLECULE	BONE FUNCTION	VASCULAR ROLE
OSTEOPROTEGERIN (OPG)	inhibits osteoclast differentiation and function	inhibits vascular calcification in animal models; acts as a marker of cardiovascular disease
SCLEROSTIN (SOST)	regulator of osteoblast function, inhibits bone formation (87)	expressed in vascular calcifications (88)
OSTEOPONTIN (OPN)	stimulator of adhesion of osteoclasts to the bone matrix	modulator of ectopic calcification
BONE MORPHOGENETIC PROTEINS (BMP)	regulator of osteoblast function, increases collagen synthesis	induces endothelial dysfunction
MGP	inhibits bone mineralization	inhibits ectopic calcification
FETUIN A	inhibits bone mineralization (89)	favours the persistence of circulating minerals preventing their ectopic deposition (89)
VITAMIN K	protective effect on bone mass	reduces the progression of vascular calcification
PHOSPHATE	key element in the bone structure	induces vascular calcification
KLOTHO (KL)	responsible of phosphate metabolism (90)	exert a direct beneficial vascular action (90)

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CATEPSIN	regulator of osteoclast function and degradation of the bone matrix proteins	contributes to atherogenesis
PARATHYROID HORMONE (PTH)	chronic and continuous high secretion of PTH inhibits osteoblast activity and increases bone resorption; intermittent administration of PTH increases bone formation	induces vascular calcification
VITAMIN D	regulator of calcium metabolism	deficiency and high doses of vitamin D promote vascular calcification
DYSLIPIDEMIA	inhibits osteoblast differentiation	promotes vascular calcifications
RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM (RAAS)	angiotensin II activates osteoclasts, stimulating bone resorption	promotes atherosclerosis

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2.2 THE RESEARCH PROJECT 1

2.2.1 Introduction

Frailty and cardiovascular disease share similar risk factors and pathophysiological progression (91). When both frailty and CVD are present, they are thought to exacerbate each other (92). Recently, large pooled data sets identified how frail individuals are at increased risk for CVD (93, 94); this relationship is also bidirectional as individuals with a high CVD risk were found to have an increased risk for incident frailty over a 4 years period (odds ratio 2.15; 95% CI confidence interval 1.68–2.75) (95). In addition, chronic inflammation and hormonal changes, which are typical of ageing are two of the pathological mechanisms (already mentioned earlier) involved both in frailty and in atherosclerosis (96).

Atherosclerosis is a well characterized chronic arterial disease and a major cause of vascular death (97). In the last decades, research efforts have focused on the identification of novel markers and potential mediators of atherosclerosis in order to provide additional insights in the pathogenesis of atherosclerotic cardiovascular disease beyond its classical risk factors (98).

Many pre-clinical and clinical evidence suggest an interplay between atherosclerosis and osteoporosis (63, 99). Since this association was hypothesized, several cross-sectional and longitudinal studies have identified an inverse relationship between atherosclerotic calcifications, cardiovascular disease and bone mineral density (62, 63, 99, 100) implying that bone metabolism may have an emerging role in atherosclerotic disease.

Heterogeneous approaches have been adopted to investigate the coexisting bone fragility and cardiovascular risk and different kind of population have been studied. Several mechanisms involving osteoblasts, osteoclasts, osteocytes and modified structural properties of the bone tissue have been explored in patients with diabetes (101). Diabetes, in fact, increases both the risk of cardiovascular disease and of osteoporotic fractures. However, very few studies have been conducted in people without diabetes.

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In this scenario, bone turnover biomarkers have been studied for a possible role in the bone-vascular axis and, therefore, as novel markers of vascular health (102, 103). This might be the first step in order to identify the markers of dysregulation in bone-vascular cross-talk which can influence the progression to frailty.

Soluble osteopontin (OPN), an abundant bone-matrix glycoprotein, has been suggested to act as a modulator of ectopic calcification, including vascular calcification (104). Of note, some studies found significant associations of OPN levels with cardiovascular disease in people with diabetes (105, 106). Osteoprotegerin (OPG), a master regulator of osteoclast differentiation and function, was shown to inhibit vascular calcification in animal models (107). Klotho (KL), an enzyme responsible of phosphate metabolism, was shown to exert a direct beneficial vascular action, as suggested by rodent models with targeted deletion of KL displaying both severe osteoporosis and progressive atherosclerosis (90). Furthermore, it has been demonstrated that sclerostin (SOST), a key element in bone resorption, is expressed in vascular calcifications (88, 108) with different levels among patients with various types of diabetes and baseline vascular risk (109, 110).

Taken together, these data encourage further research to identify biomarkers implicated in the bone-vascular axis that might significantly improve the prediction of atherosclerotic cardiovascular disease. In particular, although previous data suggest the existence of a bone-vascular axis, it is unclear whether bone biomarkers are helpful to early identify subjects with critical atherosclerosis.

2.2.2 Aim of the study

In this study, we primarily aimed to evaluate the association between serum concentrations of soluble bone biomarkers and atherosclerotic disease severity assessed through coronary angiography, gold standard for the diagnosis of coronary artery disease, and carotid Doppler ultrasound. Then, we tested the associations of these candidate bone biomarkers with the traditional cardiovascular risk factors.

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2.2.3 Materials and methods

Study design and population

This cross-sectional observational study enrolled consecutive patients undergoing elective diagnostic coronary angiography according to the treating physician indication and carotid Doppler ultrasound at the Department of Cardiovascular Sciences from September 2014 to January 2015, as previously described (111).

Briefly, participants were overweight/obese Caucasian adults with a body mass index (BMI) between 25.0 and 45.0 Kg/m^2. Exclusion criteria were history of myocardial infarction or stroke, diabetes, acute illnesses, severe systemic diseases, any clinical disorder of bone metabolism, hormone therapy or treatment with other drugs known to influence bone metabolism.

Study procedures and biochemistry

Coronary angiography was performed by experienced operators who assessed percent diameter stenosis by visual estimation. In all patients common carotid intima-media thickness (cIMT) was measured by Doppler ultrasound using B-mode ultrasonography. The mean value of 3 repeated measurements of the cIMT 1 cm distal to the bulb was reported. Plaques were diagnosed by the presence of hyperechogenic images inside the artery. The same operator made all the study measurements.

Blood was collected in all participants before coronary angiography for both routine biochemistry analyses and measurement of the following selected bone turnover biomarkers: SOST, KL, OPN and OPG. Blood was collected after overnight fast; serum was divided from plasma by centrifugation and stored at -20°C until the assays was performed. Routine biochemical panel included glucose values, lipid profile, serum creatinine and high-sensitivity C-reactive protein. Estimated glomerular filtration rate (eGFR) was calculated with CKD-EPI equation. Bone turnover biomarkers were measured from sera using enzyme-linked immunosorbent assay (ELISA). All samples were analyzed in duplicates. If duplicate results differed by more than 20%, measurements were repeated on a second aliquot. Controls were analyzed at the beginning and at the end of each plate. All analyses were performed according to the manufacturer's recommended protocol. The sensitivity of the ELISA kit used for measurement of SOST (Biomedica, Wien, Austria) was 0.079 ng/ml, in-between-run coefficient of variability (CV) was 17.5% % and in within-run CV was between 4

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and 8%. ELISA commercial kit (CUSABIO□, Houston, TX 77054, USA) used for KL had a sensitivity of 0.039 ng/ml, an intra-assay CV between 4% and 10%. OPN was analyzed by ELISA kit (R&D system, Minneapolis, MN 55413, USA) with a sensitivity of 0.011 ng/ml, intra-assay CV between 4% and 8%. OPG from sera was detected by immunoassay (R&D system, Minneapolis, MN 55413, USA); the sensitivity was 0.08 pmol/l, intra-assay CV was between 4 and 6%, while the inter-assay CV was 2.9%.

Advanced atherosclerosis was defined as a critical coronary (≥70%) and/or carotid stenosis (≥50%). The study was performed in accordance with the Declaration of Helsinki and the protocol was approved by the institution's ethics committee with all patients giving written informed consent to participate.

Statistical analysis

Results are expressed as mean ± standard deviation (SD) or median [25^{th}-75^{th} percentile] for continuous variables, as appropriate, and as percentage for categorical variables. Shapiro-Wilk normality test was used to assess the normality of continuous variables distributions (variables with Shapiro-Wilk statistic <0.9, p values <0.05 were considered non-normally distributed). Comparisons between groups were done using Student's t-test, Kruskal–Wallis, Chi-square and Fisher's exact test depending on distribution. Generalized linear models were used to adjust for confounders. Variables that showed an association with the outcome at a nominal p value of <0.10 in 2-way analysis were tested in the model for significance with the main effect and outcome variable. Nonparametric variables were natural log-transformed before testing in the model. The primary end points of this analysis (differences in KL, SOST, OPN and OPG between groups with and without critical atherosclerosis) were tested at a Bonferroni adjusted for multiple testing alpha value <0.0125, with 80% power for an effect size of 0.75. If any difference was significant, the alpha value could be recycled to test the other primary outcomes as previously described (112). A p value <0.05 was considered as statistically significant for comparison of clinical and biochemical features between groups. All statistical analyses were performed using *Stata/IC 12.1* software (StataCorp, College Statio, Texas, USA).

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2.2.4 Results

Population features

The study included 80 patients with a mean age of 68±10 years, 32.5% of whom were females. Advanced atherosclerosis was detected in 55 patients (68.5%): 30 (37.5%) had critical coronary artery disease, 7 (8.8%) had critical carotid atherosclerosis and 18 (22.5%) had both. Population features according to the presence of advanced atherosclerosis are summarized in Table 2.

TAB 2. *Population features according to the presence of advanced atherosclerosis.*
*Continuous variables are presented as mean (SD) or as median [25th-75th percentile] as appropriate. Categorical variables are represented as percentages. *p>0.05 after adjustment for statin use.*

	Advanced atherosclerosis		
	Yes (n=55)	**No (n=25)**	**P-value**
Age, years	70.0 (9.8)	65 (10)	0.036
Male gender	76.4%	45.0%	0.012
Smokers	20.0%	32.0%	0.24
Hypertension	81.8%	64.0%	0.083
BMI, Kg/m^2	26.7 (3.4)	26.1 (2.7)	0.44
Waist-to-hip ratio	0.98 [0.91-1.00]	0.96 [0.88-1.00]	0.28
Waist circumference, cm			
- **Male**	98.9 (9.3)	98.5 (9.7)	0.88
- **Female**	97.7 (10.0)	91.6 (10.1)	0.13
Fasting plasma glucose mg/dl	94 [85-99]	89 [82-102]	0.22
Total cholesterol, mg/dl	156 [133-182]	184 [159-228]	0.002*
HDL cholesterol, mg/dl	43 [36-56]	54 [45-68]	0.017*
LDL cholesterol, mg/dl	92 [74-113]	119 [96-154]	0.003*
Triglycerides, mg/dl	116 [87-177]	97 [67-148]	0.15
eGFR, ml/min/1.73^2	71.1 [57.9-90.1]	73.3 [58.3-93.5]	0.51
C-Reactive Protein , mg/l	1.34 [0.00-4.44]	0 [0.00-3.34]	0.44
IMT, mm	1.15 [0.8-1.3]	0.8 [0.8-1.1]	0.012

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Statin	73.5%	28.0%	<0.001
ASA	75.5%	44.0%	0.006
Clopidogrel	20.8%	8.0%	0.21
ACE-inhibitors	43.4%	32.0%	0.34
ARB	22.6%	24.0%	0.89
Beta-blockers	35.9%	20.0%	0.16

Patients with advanced atherosclerosis were older (70±9 vs 65±10 years, p=0.036) and more frequently males (76.4% vs 45.0%, p=0.012). Hypertension was highly prevalent in both groups (81.8% vs 64.0%, p=0.083) and no difference in smoking habits was found (p=0.24). As well, groups were similar in BMI, waist-to-hip ratio, blood glucose values and eGFR. Patients with advanced atherosclerosis were more likely to be treated with statins (73.5% vs 28.0%, p<0.001), which resulted in a more favourable lipid profile, as shown by the lower total cholesterol (156 [133-182] vs 184 [159-228] mg/dl, p=0.002; $_{adj}$p after adjustment for statin use 0.18) and LDL-cholesterol (92 [74-113] vs 119 [96-154] mg/dl, p=0.003, $_{adj}$p=0.28) values. Acetylsalicylic acid (ASA) was more frequently used in patients with critical atherosclerosis, while no difference in the use of clopidogrel, ACE-inhibitors, angiotensin receptor blockers (ARBs) and of beta-blockers was detected. Levels of OPG, OPN and KL did not differ between males and females, however SOST values were significantly higher in men than in women (40.9 [31.2-55.0] vs 32.9 [26.2-41.7] pmol/l, p=0.009).

Age was significantly and directly related with all SOST (rho=0.347, p=0.0017), OPG (rho=0.624, p<0.001), OPN (rho=0.398, p<0.001) and KL (rho=0.477, p<0.001) serum concentrations.

Bone biomarkers and advanced atherosclerosis

Compared to patients without, those with advanced atherosclerosis showed higher serum levels of OPG (5.32 [4.34-7.43] pmol/l vs 4.33 [2.90-5.05] pmol/l, p=0.002) and SOST (41.7 [31.1-53.0] pmol/l vs 32.5 [25.8-38.3] pmol/l, p=0.017). No

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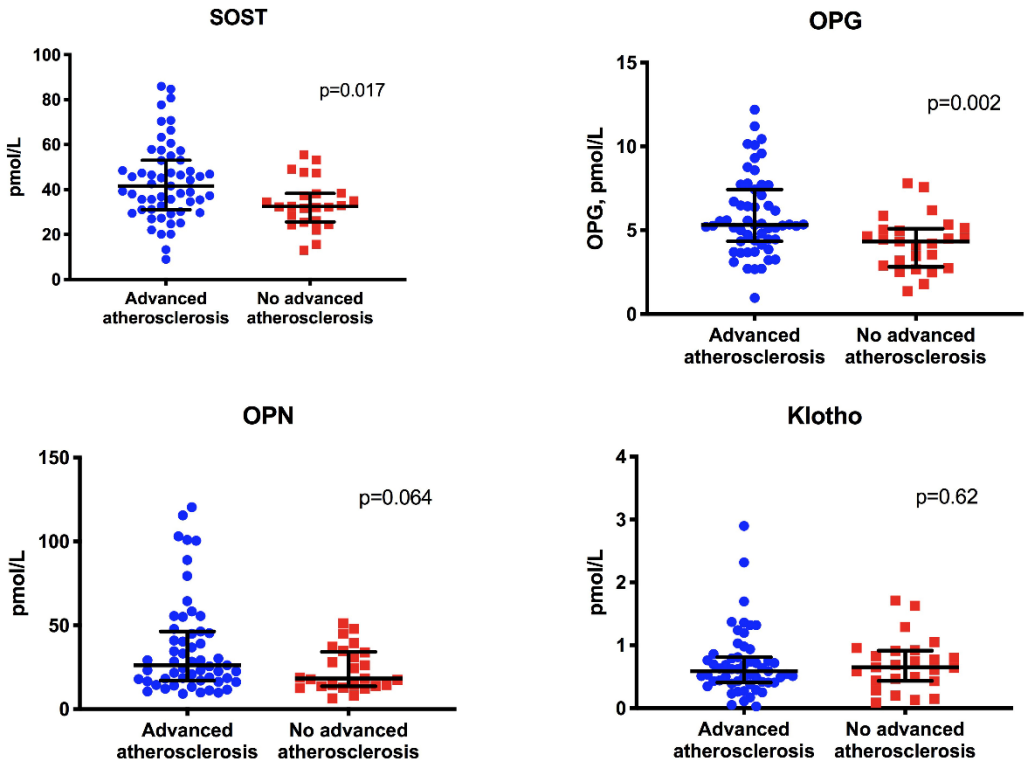
significant differences in OPN (26.2 [17.1-46.3] ng/ml vs 18.3 [13.8-33.8] ng/ml p=0.064) and KL (0.59 [0.41-0.81] ng/ml vs 0.65 [0.44-0.91], p=0.622) were observed between groups (**Fig. 9**).

FIG. 9 *Association between bone biomarkers and advanced atherosclerosis*



The difference found in OPG levels remained significant after adjustment for possible confounders (age, sex, hypertension, statin use) ($_{adj}$p value=0.006), while SOST was no longer associated with advanced atherosclerosis after adjustment for age and sex ($_{adj}$p value=0.13).

Bone biomarkers and cardiovascular risk factors

Similarly, the serum concentration of all osteokines significantly increased in parallel with declining of eGFR values (p=0.017 for SOST, p=0.001 for OPG and p<0.001 for OPN and KL) (Table 3).

KL serum levels were inversely associated with triglycerides (rho=-0.27, p=0.016) and directly related to HDL cholesterol (rho=0.29, p=0.011). Serum concentrations of OPN were directly associated with fasting plasma glucose (rho=0.38, p<0.001). An inverse association between OPN and OPG concentrations

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with LDL cholesterol was found, which however disappeared after adjustment for statin use. No other significant associations between bone biomarkers and traditional cardiovascular risk factors were found (Table 3).

TAB 3. *Correlations between bone biomarkers and cardiovascular risk factors.*
**p>0.05 after adjustment for statin use*

CV risk factor	Bone biomarkers	Rho	p-value
Age, years	**Sclerostin,** pmol/l	0.347	0.0017
	Osteoprotegerin, pmol/l	0.624	<0.001
	Osteopontin, ng/ml	0.398	<0.001
	Klotho, ng/ml	0.477	<0.001
BMI, Kg/m^2	**Sclerostin,** pmol/l	-0.015	0.892
	Osteoprotegerin, pmol/l	0.009	0.938
	Osteopontin, ng/ml	-0.043	0.706
	Klotho, ng/ml	-0.119	0.291
Waist-to-hip ratio	**Sclerostin,** pmol/l	0.197	0.090
	Osteoprotegerin, pmol/l	0.036	0.760
	Osteopontin, ng/ml	0.196	0.090
	Klotho, ng/ml	-0.083	0.474
Fasting plasma glucose, mg/dl	**Sclerostin,** pmol/l	0.195	0.086
	Osteoprotegerin, pmol/l	0.202	0.072
	Osteopontin, ng/ml	0.380	<0.001
	Klotho, ng/ml	0.120	0.289
Total cholesterol, mg/dl	**Sclerostin,** pmol/l	-0.077	0.507
	Osteoprotegerin, pmol/l	-0.395	<0.001
	Osteopontin, ng/ml	-0.319	0.004*
	Klotho, ng/ml	-0.085	0.460
HDL cholesterol, mg/dl	**Sclerostin,** pmol/l	-0.140	0.225
	Osteoprotegerin, pmol/l	0.031	0.785
	Osteopontin, ng/ml	-0.181	0.112
	Klotho, ng/ml	0.285	0.011
LDL cholesterol, mg/dl	**Sclerostin,** pmol/l	0.012	0.915
	Osteoprotegerin, pmol/l	-0.367	0.001*
	Osteopontin, ng/ml	-0.306	0.007*
	Klotho, ng/ml	-0.193	0.091
Triglycerides, mg/dl	**Sclerostin,** pmol/l	0.060	0.603
	Osteoprotegerin, pmol/l	-0.197	0.084
	Osteopontin, ng/ml	0.037	0.750
	Klotho, ng/ml	-0.271	0.016*
C-reactive protein, mg/l	**Sclerostin,** pmol/l	0.005	0.969
	Osteoprotegerin, pmol/l	0.020	0.859
	Osteopontin, ng/ml	0.075	0.515
	Klotho, ng/ml	0.162	0.157

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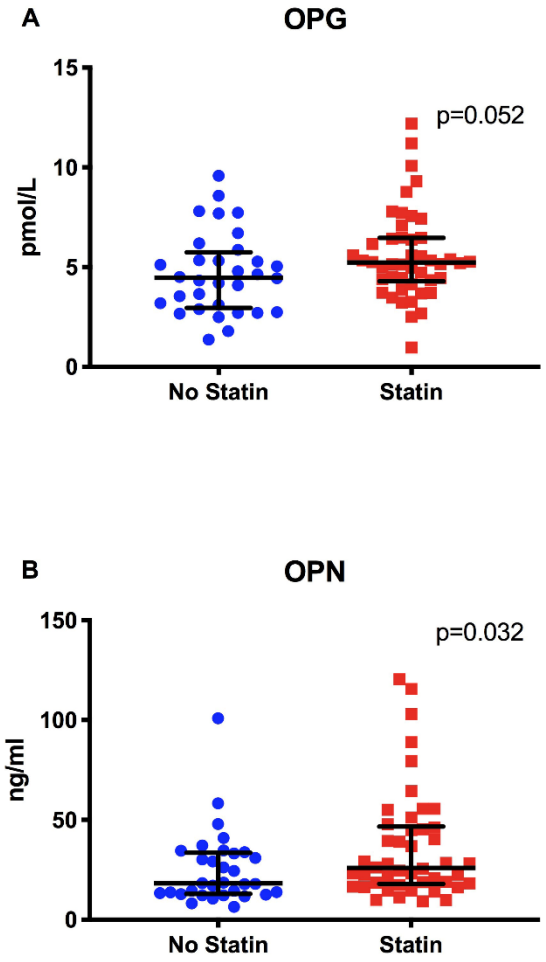
eGFR, ml/min/1.73m^2	**Sclerostin,** pmol/l	-0.263	0.022
	Osteoprotegerin, pmol/l	-0.415	<0.001
	Osteopontin, ng/ml	-0.377	<0.001
	Klotho, ng/ml	-0.540	<0.001

Patients treated with statins showed higher values of OPN (25.9 [18.1-46.3] pmol/l vs 18.1 [13.1-33.5] pmol/l, p=0.032) and OPG (5.23 [4.34-6.47] pmol/l vs 4.47 [3.01-5.61] pmol/l, p=0.052) compared to patients who did not use statins (Fig. 9). Statins use was not associated with serum levels of SOST and KL.

FIG. 10 *Difference in OPG (panel A) and OPN (panel B) levels in patients treated or not with statins*



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2.2.5 Discussion

In this study we found that overweight/obese patients presenting with critical coronary and/or carotid atherosclerotic stenosis had higher serum concentrations of OPG compared to participants without advanced atherosclerosis, independently from confounders (age, gender and use of statins). Furthermore, increased levels of KL were more frequently observed in patients at high cardiovascular risk, such as those with low HDL and high triglycerides values. However, neither KL, OPN or SOST were independently associated with clinically critical atherosclerotic plaques.

Our findings reinforce and expand previous data supporting the existence of a bone-vascular axis and the potential role of bone biomarkers for cardiovascular risk stratification.

A possible role for OPG in atherosclerotic disease is supported by both preclinical and clinical models. However, it is still debated whether the expression in vascular lesions of molecules involved in the OPG/RANKL/RANK axis, as well as serum levels of OPG, attenuate cardiovascular disease or are instead representative of a counter-regulatory protective pathway activation in the context of vascular calcification processes. In this regard, OPG-knockout mice have concomitantly shown early onset osteoporosis and increased vascular calcifications (113), while, on the other hand, observational studies in humans suggests the association of higher OPG levels with the presence of vascular calcifications and increased risk of cardiovascular mortality (114-118).

Increased levels of SOST have been described both in bone specimens of patients at high cardiovascular risk, such as those with type 2 diabetes, compared to non-diabetic peers, and, in the sera of patients with type 2 diabetes and increased IMT or aortic calcifications compared to people with type 2 diabetes but without signs of macrovascular disease (119, 120). Consistently, we found that serum SOST concentrations are increased in non-diabetic patients with advanced atherosclerotic disease in the coronary or carotid arteries. However, our data also suggest that this association may be mediated by aging, a major non-modifiable cardiovascular risk factor, de-escalating the potential role of this molecule as independent biomarker for cardiovascular risk prediction. Indeed, SOST might still have a potential role in the pathogenesis of vascular disease, as corroborated by a recent study showing that

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vascular smooth muscle cells may acquire an osteocyte phenotype able to produce SOST (119). Additionally, the use of Romosozumab, a monoclonal anti-SOST antibody for the treatment of post-menopausal osteoporosis was associated with an increased risk of cardiovascular adverse events, further corroborating a key role of SOST in the pathogenesis and stability of atherosclerotic plaques (121).

The relationship we observed between KL and traditional cardiovascular risk factors, such as HDL cholesterol and triglycerides, is in line with the evidence that KL is able to stimulate insulin sensitivity and keep circulating fatty acids in balance (122, 123). Klothos exists in two forms, encoded by different genes, that activate circulating fibroblast growth factors (FGF19, FGF21 and FGF23). α-klotho is required for FGF23-dependent signalling, involved in phosphate and calcium homeostasis, whereas β-klotho is essential for FGF19- and FGF21-dependent signalling, that respectively regulates bile acid synthesis and lipogenesis and stimulate insulin sensitivity, energy expenditure and weight loss (124). In this study we specifically measured α-klotho, which has also been found to improve glycogen synthesis and storage and suppresses hepatic gluconeogenesis and lipogenesis by elevating hepatic insulin sensitivity (122). Furthermore, obese mice treated with α -Klotho showed augmented lean mass, reduced adiposity and raised energy expenditure, in addition lipid accumulation in liver and adipose tissue was also reduced compared to controls (123). Despite the fact that we did not observe a significant difference in the serum concentration of KL between subjects with and without advanced atherosclerosis, KL has been shown to contribute to the maintenance of endothelial integrity and to exhibit anti-inflammatory properties by suppressing TNF-alpha expression (125). A possible explanation of this inconsistency may be that these activities are particularly relevant in the early stages of atherosclerotic disease, which may not be explored in our high-risk population. Further studies should elucidate the role of KL as possible biomarker or mediator of early atherosclerosis.

In contrast with previous findings, in this study serum OPN levels were found to be nominally but not significantly higher in patients with advanced atherosclerosis.

Finally, in this study we put in evidence the direct association between serum concentrations of OPG and OPN and the use of statins. This finding is in line with the osteo-anabolic and antiresorptive properties of statins suggested by *in-vitro* and *in-*

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vivo evidence (126, 127). However, a meta-analysis including observational and randomized studies reported a trend towards fewer hip fractures among statin users in four prospective cohort studies but this trend was not confirmed in the two randomized trials looking at femoral and vertebral fractures (127).

One limit of this study is the lack of information concerning frailty assessment. In the future it will be useful to establish the potential role of osteokines as a marker of frailty and its progression. Furthermore, the sample size providing enough power to detect large, but not small, effect sizes, and the inclusion of patients with a clinical indication for an invasive coronary diagnostic test, which results in the selection of a population at high risk for cardiovascular disease. Therefore, our findings may not apply to a lower risk community.

The selected bone biomarkers have so far been examined in diabetes and chronic kidney disease (107, 128). This is because both diabetes and chronic renal failure increase the risk of cardiovascular disease and osteoporotic fractures. The main strengths of the research project are related to the fact of being the first study conducted on a population with atherosclerosis and at high risk of cardiovascular disease but without a personal history of diabetes and/or CKD; and from the evaluation of four candidate biomarkers reflecting different axes of bone metabolism selected on the basis of previous evidence suggesting a possible role in the pathogenesis of atherosclerosis.

In conclusion, this study conducted in a high-risk population of overweight-obese patients without diabetes demonstrates that elevated serum levels of OPG are independently associated with advanced atherosclerosis, confirming a common bond between bone metabolism and vascular disease. Further investigations on the role of selected bone biomarkers in the pathogenesis of cardiovascular disease and for risk prediction are needed.

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CHAPTER 3

RESEARCH PROJECT 2: *Vertebral fractures as markers of frailty in very-high cardiovascular risk population*

3.1 BONE HEALTH AND FRAGILITY FRACTURES

3.1.1 Background

Osteoporosis is a condition resulting in significant morbidity and mortality. Every year about 8.9 million fragility fractures occur in the world (129). Despite this incidence, osteoporosis is frequently untreated.

Vertebral fractures often represent the first signs of osteoporosis, but only one third of the subjects who are affected by a fracture will have a clinical diagnosis (130). Sometimes these fractures are asymptomatic, other times they are associated with back pain and decreased activity, thus they are generally ignored by patients and physicians or are attributed to degenerative joint disease, especially in elderly people (130). However, the presence of a vertebral fracture helps to predict the risk of new fractures and may influence the choice for a specific treatment.

Bone mass is influenced by genetic and hormonal factors (endogenous) and by nutritional factors and features related to physical activity (exogenous). The main mineral component of the human body is calcium; the 99% of calcium which is present in our body is contained in an insoluble form in the bone tissue. Here calcium strengthens the skeleton, supports locomotion and helps protecting the internal organs (131). Intuitively bone health can be said to depend on nutrition and in particular it is related to calcium and vitamin D. Low calcium intake is associated with a reduced bone mass and osteoporosis, whereas a severe vitamin D deficiency leads to a decreased bone mineralization which is called osteomalacia. The SENECA (Survey in Europe on Nutrition and the Elderly) study evaluated the daily average intake of calcium in 10 European countries: it revealed a very low dietary calcium intake in about one third of the individuals, as well as vitamin D insufficiency in the majority of elderly people enrolled (132). The best way to reach an adequate calcium intake is through diet. Only when the dietary source is not enough calcium supplements can significantly support the intake. Indeed, recent studies showed the positive effect of

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calcium and vitamin D treatment not only for bone mass but also for fractures incidence (133, 134). Thus supplements are a valid cheap and safe means of prevention of fragility fractures.

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3.1.2 Clinical significance and diagnosis of vertebral fractures

Osteoporotic vertebral fractures increase the risk of other major fragility fractures, both vertebral and non-vertebral, besides being responsible for high health costs and mortality (135). They represent the most common osteoporotic fractures worldwide and they occur in 30-50% of the population over 50 years (136). Unlike hip fractures vertebral fractures are often clinically silent and only about 10% require hospitalization (136).

As osteoporotic fractures their incidence rates rise with age; the rates also change according to the method used to define the vertebral fracture. Furthermore, it is important to reinstate how nowadays radiologists often do not report the presence of vertebral fractures on the spine radiograph, these records are thus often missed in the medical anamnesis and rarely they are thought to deserve specific treatment (136).

As indicated by the osteoporosis guidelines, both diagnosis and the correct classification of vertebral deformity are identified through semi-quantitative (SQ) or quantitative vertebral morphometry (137). Depending on the type and severity of the reduction in spinal height, X-ray studies allow to identify three types of vertebral fractures: wedge-shaped (anterior), biconcave (middle) and total vertebral collapse. SQ methods are based on a first visual evaluation of images of the spine for a differential diagnosis of vertebral deformities, followed by a visual gradation of the osteoporotic vertebral fractures that are considered mild, moderate, or severe (Genant criteria) (Fig. 11) (138). Vertebral morphometry – based on the measurement of vertebral height – is carried out on the images of lateral projections of the thoraco-lumbar spine through the use of DXA (MXA) or conventional radiology (MRX), using VFA software (vertebral fracture assessment) (130).

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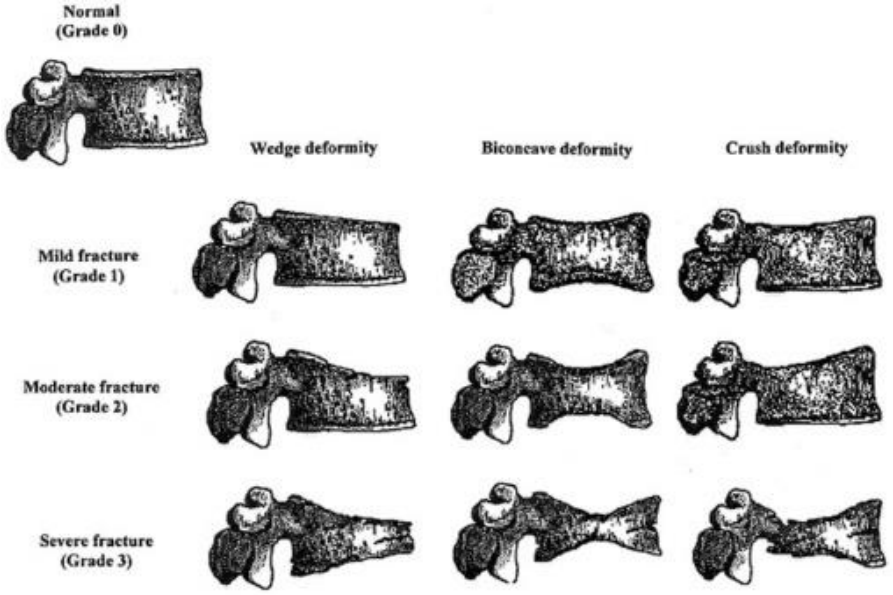
FIG. 11 *Genant semi-quantitative grading scale for vertebral fractures (138).*



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3.1.3 Bone physiology

Bone is a dynamic organ in which the action of bone cells plays a crucial role in bone remodeling. Physiological bone remodeling is linked to the balance among different cells: osteoclasts, which reabsorb bone tissue; osteoblasts, which secrete osteoid and modulate crystallization of hydroxyapatite; osteocytes, which occupy a small chamber called lacuna, contained in the calcified matrix of bone, and which are further responsible of the communication among bone cells; finally bone lining cells, whose function is not well known (139).

Several bone diseases are caused by the imbalance between the formation and reabsorption of bone tissue. For example, osteoporosis is caused by an excessive osteoclasts reabsorption compared to osteoblasts formation, which results in bone loss, rise of fragility and risk of fractures.

There are two types of bone:

- cortical bone: it forms the surface of most bone and the shafts of the long bones. It is compact and thick. The 80-90% of it is calcified and it has a structural function (140).
- trabecular bone: it is found inside flat bones, vertebrae and in the end of long bones; it is similar to a sponge. Only 15-25% of it is calcified and it has a metabolic role (140).

Bone is a tissue consisting of about 70% mineral and 30% organic constituents. The organic constituents are 98% collagen fibres, glycoproteins and proteoglycans. The mineral composition is about 95% hydroxyapatite, organized crystal of calcium and phosphorous, and other ions. As evidence shows, calcium is the main component of the bone structure and it strengthens the structure of the skeleton. At more than 99% it is present as calcium-phosphate complexes, thus the bone represents a calcium pool that is useful to maintain the intra- and extra-cellular calcium balance (131).

Calciotropic hormones, PTH, 1,25-dihydroxyvitamin D [1,25(OH)2D], Fibroblast Growth Factor 23 (FGF23) and calcitonin contribute to maintaining the concentration of serum calcium. A reduction of serum calcium increases the production of PTH from parathyroid glands. PTH induces the release of calcium from bone; in kidneys it increases the tubular calcium reabsorption and stimulates the secretion of [1,25(OH)2D], which activates the vitamin receptor D (VRD) in gut and increases

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calcium absorption. Increases in serum calcium concentrations are contrasted by calcitonin, produced by the thyroid gland, through a negative feedback by [1,25(OH)2D] on PTH secretion. FGF23 regulates the phosphate serum levels and indirectly the calcemia.

To sum up, the mechanisms triggered by the calciotropic hormones are necessary to preserve calcium homeostasis in healthy subjects.

Lastly it is important to specify that bone density is determined by genetics, hormones and environmental factors, in particular physical activity and nutrition.

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3.1.4 Calcium intake from food

The adequate assumption of bone nutrients is one of the most important ways to preserve the optimum mass and density of bone during adulthood. Calcium and vitamin D have been demonstrated to be essential for normal bone growth and for the maintenance of bone mineral density in postmenopausal women (141). An optimal calcium intake is needed in all stages of life, as suggested by the Reference Levels of Nutrients and energy intake for the Italian population (LARN) (142) (Table 4).

Milk and dairy products (yogurt, cheese) are the main sources of calcium, yet several vegetables as well as natural mineral water can also provide calcium.

TAB. 4 *Recommended average daily requirement (142).*

		Ca (mg)
LATTANTI	6-12 mesi	nd
BAMBINI-ADOLESCENTI		
	1-3 anni	500
	4-6 anni	700
	7-10 anni	900
Maschi	11-14 anni	1100
	15-17 anni	1100
Femmine	11-14 anni	1100
	15-17 anni	1000
ADULTI		
Maschi	18-29 anni	800
	30-59 anni	800
	60-74 anni	1000
	≥75 anni	1000
Femmine	18-29 anni	800
	30-59 anni	800
	60-74 anni	1000
	≥75 anni	1000
GRAVIDANZA		1000
ALLATTAMENTO		800

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3.2 THE RESEARCH PROJECT 2

3.2.1 Introduction

The current growth of the ageing population is connected both to an increasing number of frail elderly people and to the rising incidence of degenerative diseases such as cardiovascular diseases and osteoporosis. (143). In Italy for example the impact of osteoporotic hip fractures is highly similar to that of acute myocardial infarction (AMI) (143). According to the World Health Organization (WHO) cardiovascular disease is the first critical health problem, while the second is osteoporosis (143).

An increasing number of biological and epidemiological investigations suggest a link between cardiovascular disease and bone biology. Traditional cardiovascular risk factors are considered to be osteoporosis risk factors. Ageing, sedentary lifestyle, smoking, alcohol consumption are some of the known environmental factors which are able to increase the risk of atherosclerosis and bone loss (144).

Many studies showed that subjects with cardiovascular diseases are more likely to develop major osteoporotic fractures. In the Swedish Twin Register ischemic heart disease was associated with a higher risk of hip fracture (145); further studies confirmed the association of heart failure with hip fractures (146, 147). Considering instead the effect of osteoporotic fractures on cardiovascular diseases, research on a group composed of postmenopausal women who had at least one severe vertebral fracture (Grade 3 according to Genant's scale) showed that this group had a fourfold higher risk of major cardiovascular events (e.g. myocardial infarction, stroke, sudden death) compared to women without vertebral fractures (138, 148).

In the past vessel calcification was considered to be merely a passive process. Nowadays the strong similarities between cells of the vascular wall and cells of bone tissue have been established, in fact some key modulators of bone and mineral metabolism are actively involved in the vascular calcification process (77, 89).

Calcium is one of the elements involved both in the development of osteoporosis and in the increase of atherosclerosis. Inadequate daily calcium intake is one of the common pathogenic pathways causing hypertension and the reduction of bone mineral density (143). Low calcium serum levels have been demonstrated to stimulate PTH

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and vitamin D production, resulting in lipogenesis carried out by adipocytes and leading to rising systolic blood pressure, atherosclerosis and cardiovascular events (143).

This knowledge encourages further epidemiological investigations and longitudinal trials which could explore the possible mechanisms shared by cardiovascular disease, osteoporosis and calcium metabolism.

3.2.2 Aim of the study

The aim of the present study was to investigate the prevalence of vertebral fractures in very high cardiovascular risk population and to evaluate a possible relationship with frailty and the daily intake of calcium from food.

3.2.3 Materials and methods

Population

This prospective observational study enrolled consecutive patients who were admitted to the Vascular Surgery Department of the Immaculate's Dermatopathic Institute (IDI-IRCSS) in Rome between the 1st of January 2018 and the 31st of March 2018. All the enrolled patients were candidates for vascular surgery (revascularization procedures or carotid endarterectomy) and each presented at least one of the cardiovascular complications needed to define the subject at very high cardiovascular risk according to the guidelines (68) (Fig. 5). The exclusion criteria were oncological diseases in progress and circumstances in which lateral chest X-ray couldn't be performed.

Study procedures and biochemistry

For each patient a dual-projection chest radiograph was carried out at the admission to the department. In all the lateral chest X-rays vertebral fractures were evaluated through a semi-quantitative method using the Genant classification (Fig. 11) (149). Vertebral morphometry was performed manually by an experienced operator, and the same operator carried out the study measurements.

On the first day of hospitalisation blood was collected from all participants for routine biochemical analysis, including complete blood count, electrolytes, lipid

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profile, transaminases, uric acid, serum creatinine, lipid profile, high-sensitivity PCR and erythrocyte sedimentation rate (VES).

Frailty was identified through a validated questionnaire by an Italian prospective cohort study called INTER-FRAIL – intercepting frailty (Fig. 12) (150). The questionnaire was designed to be self-administrated, but in this specific study it was administered by a health professional in order to limit the error.

FIG. 12 *Questionnaire items associated with physical frailty phenotype (151).*

Item
Is your sight good enough to read newspaper headings, even with glasses? [No]
Do you easily get exhausted in daily chores? [Yes]
Do you have problems with your memory? [Yes]
Did you have any falls in last 6 months? [Yes]
Do you have difficulty walking 400 m on a flat surface? [Yes]
Do you take 5+ drugs on a regular basis (daily or almost daily)? [Yes]

Following the questionnaire results, the population was classified by using Roockwood's Clinical Frailty Scale (Fig. 13) (37). A score of 3 or 4 was associated to pre-frail, 5 or more was associated to frailty.

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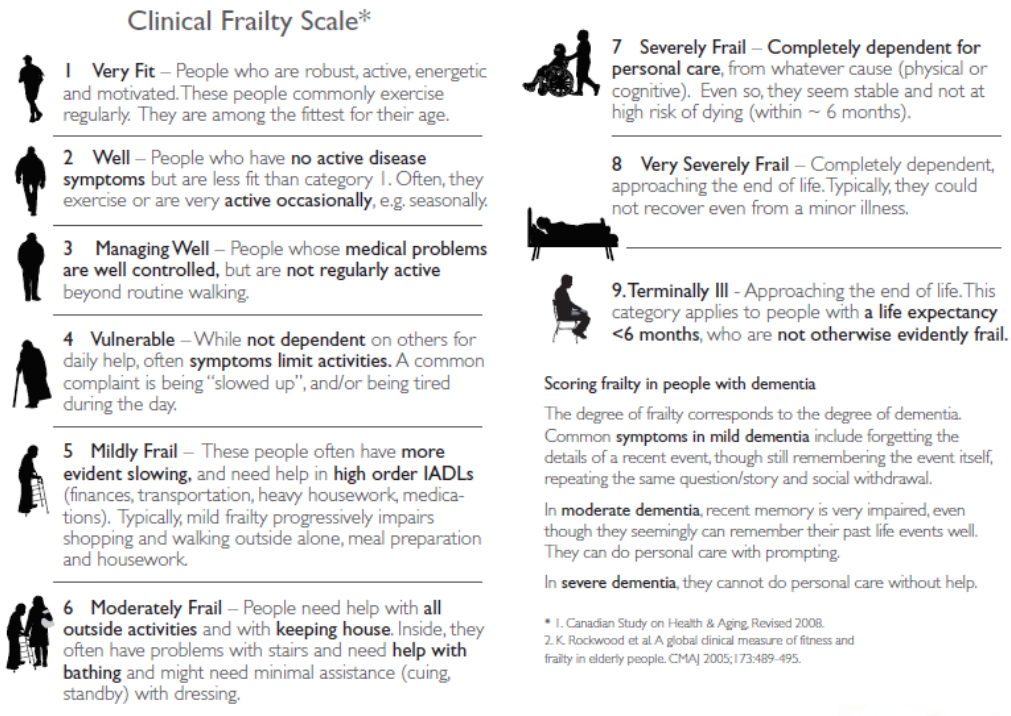
FIG. 13 *Roockwood's Clinical Frailty Scale* (34, 37).



Clinical Frailty Scale*

1 **Very Fit** – People who are robust, active, energetic and motivated. These people commonly exercise regularly. They are among the fittest for their age.

2 **Well** – People who have **no active disease symptoms** but are less fit than category 1. Often, they exercise or are very **active occasionally**, e.g. seasonally.

3 **Managing Well** – People whose **medical problems are well controlled,** but are **not regularly active** beyond routine walking.

4 **Vulnerable** – While **not dependent** on others for daily help, often **symptoms limit activities.** A common complaint is being "slowed up", and/or being tired during the day.

5 **Mildly Frail** – These people often have **more evident slowing,** and need help in **high order IADLs** (finances, transportation, heavy housework, medications). Typically, mild frailty progressively impairs shopping and walking outside alone, meal preparation and housework.

6 **Moderately Frail** – People need help with **all outside activities** and with **keeping house**. Inside, they often have problems with stairs and need **help with bathing** and might need minimal assistance (cuing, standby) with dressing.

7 **Severely Frail** – **Completely dependent for personal care**, from whatever cause (physical or cognitive). Even so, they seem stable and not at high risk of dying (within ~ 6 months).

8 **Very Severely Frail** – Completely dependent, approaching the end of life. Typically, they could not recover even from a minor illness.

9. **Terminally Ill** - Approaching the end of life. This category applies to people with **a life expectancy <6 months**, who are **not otherwise evidently frail.**

Scoring frailty in people with dementia

The degree of frailty corresponds to the degree of dementia. Common **symptoms in mild dementia** include forgetting the details of a recent event, though still remembering the event itself, repeating the same question/story and social withdrawal.

In **moderate dementia**, recent memory is very impaired, even though they seemingly can remember their past life events well. They can do personal care with prompting.

In **severe dementia**, they cannot do personal care without help.

* 1. Canadian Study on Health & Aging, Revised 2008.
2. K. Rockwood et al. A global clinical measure of fitness and frailty in elderly people. CMAJ 2005; 173:489-495.

The daily calcium intake was assessed using the Food Frequency Questionnaire (Fig. 14) (152) (which had been validated for the Italian population), while the macronutrients breakdown in a single day was investigated by collecting a 24h Recall (the percentages were evaluated as accurately as possible using the Scotti-Bassani photographic atlas). In this study the estimate of daily calcium intake was calculated using the food composition tables from the National Research Institute for Food and Nutrition (INRAN, 2013) (142). Food assessments and anthropometric parameters were collected and analysed by the same nutritionist.

The study was carried out in accordance with the Declaration of Helsinki.

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FIG. 14 *Food Frequency Questionnaire (152).*

FOOD QUESTIONNAIRE FOR THE ESTIMATION OF MEAN DIETARY INTAKE OF CALCIUM

Name:.....

Interviewer's name

age :.....

weight : ***Kg***..... **height:** ***cm***.....

Please, answer the following questions in order to define the usual serving size and how often you usually eat the listed kinds of foods. If you eat the same food many times per day (for example pasta at lunch and dinner) multiply the usual portion size for the number of meals.

1 – DO YOU DRINK **MILK** (except SOIA milk)? Yes □ No □
how much? **A**-one glass (100ml) □ **B**-one mug (250 ml) □ **C**-two mugs (500 ml) □
how many times per week? .....

2 – DO YOU EAT **YOGURT**? Yes □ No □
how much? **A**-one pottle (125g) □ **B**-two pottles (250 g) □ **C**-one wrapping of 500g □
how many times per week? .....

3 – DO YOU EAT **CHEESE**? Yes □ No □
how many times per week? ..... .

which kind?:

3.1 HARD CHEESE (Parmigiano, Grana, ...)? Yes □ No □
how much? **A**-small serving (40g) □ **B**-medium serving (80g) □ **C**-large serving (120g) □
how many times per week? .....

3.2 SEMI-HARD CHEESE (Emmenthal, Provolone, ...)?Yes □ No □
how much? **A**-small serving (40g) □ **B**-medium serving (80g) □ **C**-large serving (120g) □
how many times per week? .....

3.3 SOFT CHEESE (Mozzarellla, Stracchino, ...)? Yes □ No □
how much? **A**-small serving (40g) □ **B**-medium serving (80g) □ **C**-large serving (120g) □
how many times per week? .....

3.4 BUTTER MILK CURD? Yes □ No □
FROM SHEEP? □ FROM COW? □
how much? **A**-small serving (50g) □ **B**-medium serving (100g) □ **C**-large serving (200g) □
how many times per week? .....

4 – DO YOU EAT **PASTA** or **RICE**? Yes □ No □
how much? **A**-small serving (50g) □ **B**-medium serving (100g) □ **C**-large serving (150g) □
how many times per week? .....

- AND WITH HOW MANY TEASPOONS OF **PARMIGIANO/GRANA**?
A-none □ **B**-1 teaspoon (5g) □ **C**-2 teaspoons (10g) □ **D**-3 teaspoons (15g) □

5 – DO YOU EAT **BREAD** or similar (crackers, bread-stick, ...)? Yes □ No □
how much per day? **A**-100 g (=2 rosette) □ **B**-200 g □ **C**-300 g □
how many times per week?.....

Figure 1 *Continued.*

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6 – DO YOU EAT **POTATOES**? Yes □ No □
how many? **A-** small serving (200g=2 medium potatoes) □
B-medium serving (400g) □
C-large serving (600g) □
How many times per week?.....

7 – DO YOU EAT **MEAT** OR **FISH**? Yes □ No □
how much? **A**-small serving (100g) □ **B**-medium serving (150g) □ **C**-large serving (200g) □
how many times per week? .....

8 – DO YOU EAT **EGGS**? Yes □ No □
how many eggs per week? .....

9 – DO YOU EAT **LEGUMES** (beans, peas,…)? Yes □ No □
how many? **A**-small serving (80 g cooked – 1/3 of a tin) □
B-medium serving (150 g cooked –half tin) □
C-large serving (250 g a cooked– a tin) □
How many times per week? .....

10 – DO YOU EAT **VEGETABLES**? Yes □ No □
how many? **A**-small serving (100 g) □ **B**-medium serving (200 g) □ **C**-large serving (300 g) □
how many times per week? .....

11 – DO YOU EAT **FRESH FRUITS**? Yes □ No □
how many per week? .....

12 – DO YOU EAT **ICE CREAM with milk** (except sorbet)? Yes □ No □
how much? **A**-small serving (50 g) □ **B**-medium serving (100 g) □ **C**-large serving (150 g) □
how many per week? .....

13 – DO YOU EAT **milk or white CHOCOLATE**? Yes □ No □
how much? **A**-small serving (25 g) □ **B**-medium serving (50 g) □ **C**-large serving (100 g) □
how many times per week? .....

14 – DO YOU DRINK **TAP'S WATER**? Yes □ No □
how much per day? 0,500 l □ 0,750 l □ 1l □ 1,5 l □ 2 l □ 2,5l □
how many times per week?... ... ...

15 – DO YOU DRINK **CALCIUM RICH MINERAL WATER** (see the list) ? Yes □ No □
how much per day? 0,500 l □ 0,750 l □ 1l □ 1,5 l □ 2 l □ 2,5l □
how many times per week?……

Figure 1 *Continued.*

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- **Calcium content of each FFQ item**

FOODS	CALCIUM (mg/100g of food)
milk	120
yogurt	120
hard cheese	1263
semi-hard cheese	925
soft cheese	438
butter cow milk curd	295
butter sheep milk curd	166
pasta or rice	20
parmigiano or grana	1315
bread	14
potatoes	10
meat or fish	15
eggs	50
legumes	39
vegetables	47
fruit	20
ice cream with milk	85
milk chocolate	262

ITALIAN CALCIUM RICH WATERS	CALCIUM (mg/L)
Acetosa S.Paolo (Fonte)	325
Acetosella	216
Acqua della Madonna	208
Acqua Regina	670
Acqua Santa di Cianciano	705
Acqua Tettuccio	242
Cinciano	283
Don Carlo (Sorgente)	182
Donata	199
Ferrarelle	380
Fucoli	653
Gaudianello (Fonte Monticchio)	192
Generosa (Sorgente)	174
Imperatore (Kaiserwasser)	157
Lavaredo (Dolomiten)	299
Lete	339
Margherita (Fonte)	231
Pracastello	164
Regina (Fonte Staro)	157
S.Giacomo (Fonte di)	150
S.Lorenzo	250
S.Marco	341
S.Pellegrino	209

Figure 1 *Continued.*

Statistical analysis

Results are expressed as mean ± SD for continuous variables, and as percentage for categorical variables. The comparisons between groups were drawn using Student's t-test (two tailed) and Chi-square depending on the distribution of variables. Spearman's *r* was used to evaluate the correlation between two variables.

A p value <0.05 was considered statistically significant for comparison of clinical and biochemical features between groups. All statistical analyses were performed using Prism Software (GraphPad, San Diego, CA, USA) and SPSS 21 (IBM, USA).

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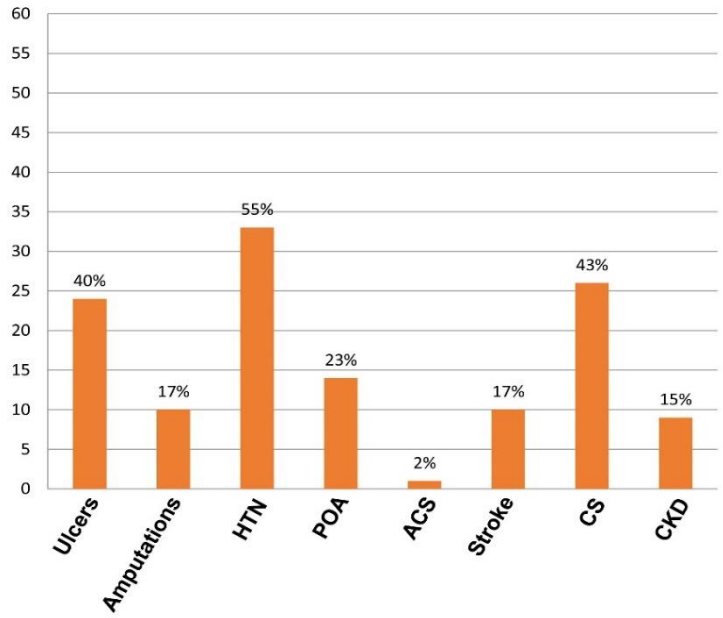
3.2.4 Results

Population features

The study included 60 patients with a mean age of 74±10 years; the 33.33% of the patients (20) were females. All patients had one or more CV complications and were in the very high-risk category (Fig. 15).

FIG. 15 *Prevalence of comorbidities. HTN: Hypertension; POA: Peripheral obliterative arteriopathy; ACS: acute coronary syndrome; CS: carotid stenosis; CKD: chronic kidney disease*.



Vertebral fractures were detected in 21 patients (35%): 9 (43%) had one vertebral fracture, 9 (43%) had two vertebral fractures and 3 (14%) had three or more fractures (Fig. 16).

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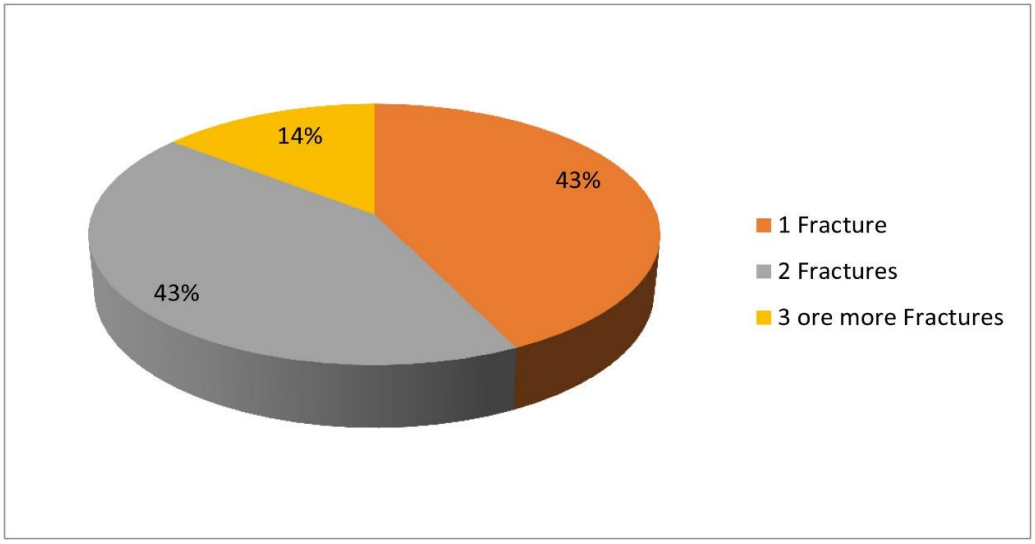
FIG. 16 *Distribution of vertebral fractures.*



The population's features according to the presence of vertebral fractures are summarized in Table 5.

TAB. 5 *Population features according to the presence of vertebral fractures.*
Continuous variables are expressed as mean ± standard deviation (SD).

	VERTEBRAL FRACTURES		**P value**
	YES (N=21)	**NO (N=39)**	
Haemoglobin , g/dl	12.25 ± 2.478	12.41 ± 2.155	0. 879
Fasting plasma glucose, mg/dl	119.05 ± 31.85	133.56 ± 63.55	0.331
HbA1c, %	7.36 ± 0.817	6.43 ± 1.327	0.258
VES	40.26 ± 34.008	41.92 ± 32.39	0.860
PCR, ml/L	37.02 ± 49.97	30.58 ± 63.81	0.696
Azotemia, mg/dl	54.43 ± 30.05	52.08 + 24.48	0.726
Creatinine, mg/dl	1.54 ± 1.23	1.57 ± 1.889	0.945
AST, U/L	19.40 ± 5.510	19.44 ± 8.778	0.984
ALT, U/L	17.35 ± 9.201	15.75 ± 6.81	0.462
GGT, U/L	28.60 ± 21.51	29.97 ± 24.29	0.834
Calcium, mg/dl	8.87 ± 0.878	8.78 ± 0.420	0.581
Phosphorus, mg/dl	2.07 ± 1.741	1.89 ± 1.828	0.714

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Magnesium, mg/dl	1.45 ± 0.647	1.21 ± 0.791	0.236
Uric acid, mg/dl	5.28 ± 2.19	5.28 ± 2.142	0.999
Total cholesterol, mg/dl	148.67 ± 56.73	161.85 ± 71.018	0.467
HDL cholesterol, mg/dl	46.60 ± 17.51	47.44 ± 14,52	0.847
LDL cholesterol, mg/dl	85.35 ± 34.048	100.69 ± 48.373	0.215
Triglycerides, mg/dl	121.90 ± 60.80	143.64 ± 84.515	0.316

Patients with vertebral fractures were older (78.19±7.85 vs 71.1±15.61, $p<0.05$). The comparison between clinical features indicates that the two groups of patients did not differ in biochemical parameters. Furthermore, data analysis from the two groups did not reveal statistical significance for any of the comorbidities present in the study population and any of the antidiabetic drugs taken.

Food calcium intake

Eating habits and macronutrient intake were investigated across 17 patients.

The daily calcium intake, evaluated through Food Frequency Questionnaire, was 784.77±255.91 mg. No significant differences in daily calcium intake (702±122.09 mg vs 783.50±277.98 mg, $p=0.1$) were observed between groups.

Frailty score

All enrolled subjects had a frailty score higher than 3, that is all patients were pre-frail or frail. Compared to patients with no vertebral fractures, those with vertebral fractures had a higher frailty score (6.67±1.39 vs 4.42±0.57, $p<0.001$).

Frailty degree was inversely associated with daily calcium intake (rho=-0.56; $p=0.019$) (Fig. 17). In addition, calcium intake was lower in frailty subjects than in those pre-frail (619,448±159,543 vs 931,738±238,907, $p=0.007$).

An inverse correlation, close to significance (rho= -0.36; $p=0.14$), was found between frailty score and the daily protein intake (Fig. 18). No other significant associations between frailty and macronutrients (lipids and carbohydrates) were found.

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FIG. 17 *Scatterplot of CFS vs Daily Calcium Intake (mg/die).*

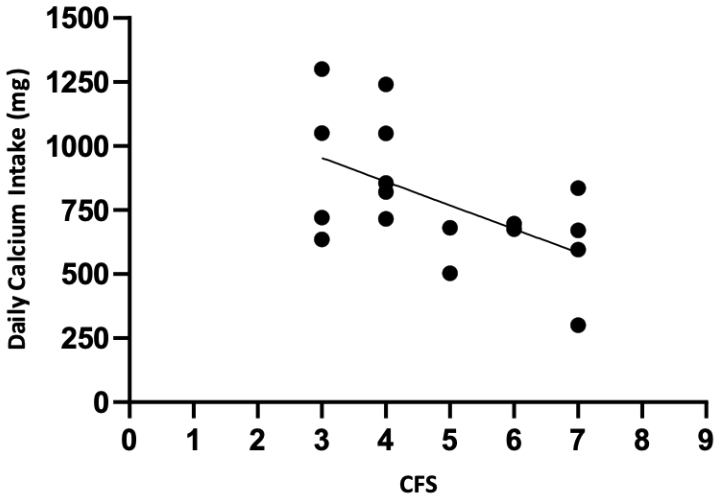
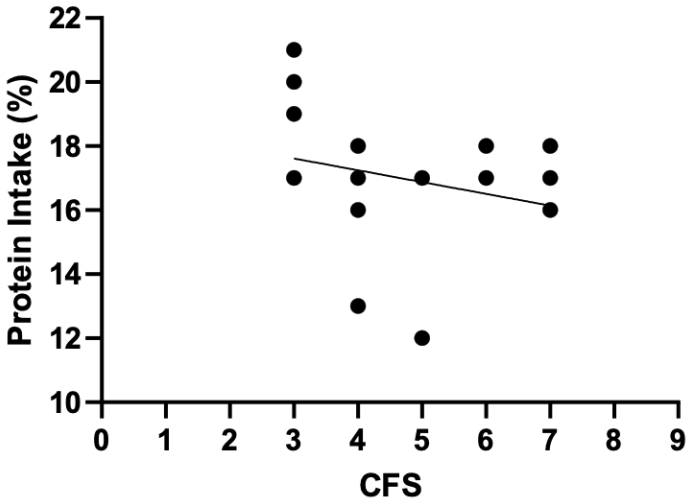


FIG. 18 *Scatterplot of CFS vs Daily Protein Intake (%).*



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3.2.5 Discussion

In this study we found that all individuals with very high-risk of CV events were pre-frail or frail and that the presence of vertebral fractures was associated with a worse state of frailty of the elderly patient. Furthermore, lower food calcium intake was more frequently observed in patients with higher CFS. Similar data was collected for daily protein intake, but this did not achieve the conventional threshold levels of statistical significance.

Our findings improved previous data, further supporting the association between risk of fracture, cardiovascular disease and frailty, besides the link to the reduction of macronutrients intake in frail people.

The study population resulted pre-frail and frail suggesting that the presence of comorbidity, in particular CV disease, may causes pre-disability.

Vertebral fractures are a sign of skeletal fragility, which indicates a higher risk of both vertebral and non-vertebral fractures after adjustment for age, BMD and other risk factors. Osteoporotic fractures often occur in the spine and vertebral fractures represent one of the components of morbidity caused by osteoporosis (135). Frailty is the functional deterioration of multiple physiological systems, a process which includes the most dramatic changes in the musculoskeletal system (2, 153). Frailty is both a consequence and a causal factor leading to fracture. Consequently, this relationship can lead to further fractures, greater frailty (154, 155) and several adverse events (2). In hospitalized patients vertebral fractures can usually be detected on lateral chest X-rays which are originally conducted for other reasons and it has long been recognized. Nevertheless, they are still not reported by radiologists and often they are not considered on a clinical level (156-159). The main result emerging from this study was the importance of performing opportunistic screening of vertebral fractures on lateral spine and chest X-ray as such screening reveals to be an untapped potential method of fracture assessment in older population and in particular in frail people. Considering this aspect, fracture prevention could be an important aspect in the improvement of ageing and frailty.

The US Dietary Guidelines Advisory Committee indicated how many nutrients are under-consumed when considering the average requirements and the adequate intake levels which have been established (160). Among the nutrients to be evaluated as

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insufficient, calcium, vitamin D, and potassium are described to be important for public health inasmuch as – according to the scientific literature (161) – their low intake has been associated with negative health effects. However the intake of milk and dairy, rich in protein and calcium, is going down in western countries (162). Insufficient intake of protein and calcium is still common in community-dwelling older adults (163). Consistently in our study we found that the daily intake of calcium was inadequate in all enrolled patients and it was lower in subjects with a frailty score equivalent to mild, moderate and severe frail than in patients with a pre-frail diagnosis.

Calcium dietary intake is recognised to be a contributing factor to the normal growth and development of bone, besides preventing bone mineral loss. The adequate consumption of food which is rich in calcium is the cheapest and safest way to prevent and improve osteoporosis. Furthermore a large cohort study from 21 countries in 5 continents revealed how a lower risk of mortality and major CVD events were related to dairy consumption (164). In addition, Gijsbers et al. conducted a meta-analysis which found out how the total intake of dairy products (especially yogurt) was inversely associated with the risk of T2D (165). Finally, Cuesta-Triana et al. researched the efficacy of dairy products intake in the prevention of frailty, sarcopenia and cognitive decline in the elderly people(166). They revealed how the intake of dairy in elderly individuals may reduce the risk of frailty; in particular the high consume of low-fat milk and yogurt, together with the addition of milk protein to regular diet, may increase skeletal muscle mass and reduce the risk of sarcopenia (166). Similarly, to previous findings, this study found the daily protein intake to be lower but not significantly so in patients with a higher frailty score.

The potential flaws of this study include the sample size which allowed us to detect large effect sizes but not the small ones, and the fact that we only investigated vertebrae from C1 to T12/L1 as often in the X-ray made for pulmonary evaluation only the thoracic vertebrae and possibly L1 are visible. Thus our findings cannot be applied to the lumbar spine.

The strongest outcomes to emerge from the study are the following: first of all the study was the first one to be conducted on a hospitalized population in a vascular surgery department; secondly the synchronous evaluation of thoracic vertebral fractures, frailty index and macronutrients intake put forward the potential role of

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opportunistic screening of vertebral fractures and of calcium intake in frail people, as well as the possible role of both atherosclerosis and osteoporosis in frailty.

In conclusion, this study conducted on a hospitalized population affected by CV complications demonstrates how unrecognized vertebral fractures and a low daily calcium intake are associated with a higher frailty score, confirming the link between bone health and vascular diseases. In order to prevent frailty, further investigations on the role of calcium metabolism in the pathogenesis of cardiovascular diseases and osteoporosis are needed.

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CHAPTER 4

RESEARCH PROJECT 3: *CT-scan model of ageing: muscle mass, bone density and vascular calcification in elderly people with SARS CoV 2 pneumonia*

4.1 CHANGES IN TISSUE DURING AGEING

4.1.1 Background

The identification of older individuals who are frail or pre-frail is a recent cornerstone of geriatric care, one that allows to apply appropriate evaluation and interventions. Despite the disagreements on a standard operational definition of frailty, significant progress has been made over the last two decades, as demonstrated by the increasing number of scientific publications on this topic. From a clinical point of view, frailty may be conceptually defined as a recognizable state in older people who suffer from increasing' vulnerability: this vulnerability results from age-associated declines in physiological reserve and function across multiple organ systems, one example is when the ability to cope with every day or acute stressors is compromised (29). While health care providers and researchers in the field of ageing have long been aware of the term "frailty", the definition and operationalization of this concept in non-specialized health settings remain elusive.

In a new scenario, the ongoing COVID-19 pandemic – caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) – has triggered a discussion on frailty. On one side strong pressure on health services and scarce resources are determining a selection based on frailty degree for patients with COVID-19 admitted to critical care; on the other, among older people frailty is a determining factor causing high vulnerability, severe clinical manifestations and death from COVID-19 (167).

Several strategies have been put forward to address evidence-practice gaps in the clinical care of frailty. Among these, there are studies which better articulate links between biological changes and changes in organs and tissue driving to frailty and vulnerability, and physiological-based studies which identify precise biomarkers of frailty (168).

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Sarcopenia is the most common physiological component of frailty. The loss of skeletal muscle and muscle strength is caused by hormonal changes related to ageing and by modifications of inflammatory pathways, including the increase of inflammatory cytokines (20).

Recently the conception of frailty as being related to osteoporosis has been increasingly accepted in studies on elderly people, in fact new research measures frailty as a predictor of osteoporotic fractures and vice versa (154).

At the same time arterial calcification has been suggested as a potential biological measure of ageing, because of the strong association existing between arterial calcification and atherosclerosis (169).

Calcium efflux from bone increases with age-related bone loss and it can reduce BMD (170). By contrast, the accumulation of age-related calcium in the arterial wall progressively stiffens blood vessels (170). However, the relations among these processes have not been further explored yet.

To sum up, sarcopenia, bone loss and vascular calcifications seem to be markers of ageing. They can also express physiological declines in multiple tissues, provoking increased vulnerability as it happens in frailty.

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4.1.2 Thoracic Aorta Calcification

The detection of subclinical atherosclerosis is one of the important challenges in vascular medicine today. It aims at identifying patients at risk of vascular events and at putting into practice possible prevention strategies. Nowadays computed tomography (CT), together with ultrasound techniques, is used to detect subclinical atherosclerosis. The assessment of coronary calcification through Agatstone calcium score on non-contrast enhanced computed tomography has been introduced as a standard method to evaluate calcification in coronary plaques. Coronary artery calcification (CAC), together with established risk factors, is a risk predictor of myocardial infarction and stroke (171).

Over the last few years CT has successfully evaluated aortic calcification, which is a highly sensitive investigator-independent marker of subclinical atherosclerosis (171). Arterial calcification of thoracic aorta is present in many elderly subjects with high susceptibility to ageing-related disease and to mortality related to non-cardiovascular disease. In 2018, Thomas and colleagues evaluated the association of thoracic aorta calcifications (TAC) with non-CVD morbidity and mortality in a longitudinal study. They demonstrated that TAC had a stronger association with non-CVD morbidity and mortality than CAC, and this included chronic obstructive pulmonary disease (COPD), hip fracture and pneumonia, thus suggesting that arterial calcification may reveal individuals with an increased vulnerability to non-CVD mortality (172) (Fig. 19).

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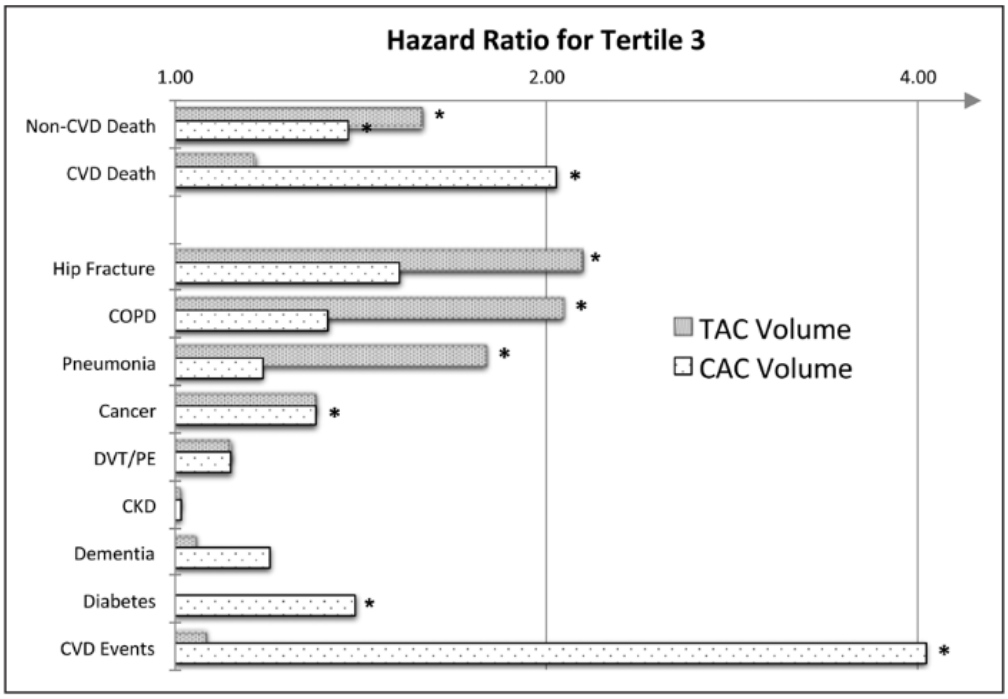
FIG. 19 *Associations of thoracic aorta calcium (TAC) and coronary artery calcium (CAC) with mortality and incident morbidities (172).* **P<0.05. CKD: chronic kidney disease; COPD: chronic obstructive pulmonary disease; DVT: deep vein thrombosis; PE: pulmonary embolism.*



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4.1.3 Computed tomography-derived bone mineral density (BMD) assessment

Central dual-energy x-ray absorptiometry (DXA) of the hips and lumbar spine is deemed to be the gold standard for diagnosing osteoporosis (137), although it is often underused. The WHO defines osteoporosis with a BMD T-score ≤ -2.5, whereas osteopenia is defined with a BMD T-score between -1 and -2.5 (137). Osteoporosis is still underdiagnosed and undertreated.

Over the last few decades several studies used BMD data obtained on CT scan which had been performed for other indications, with the aim of increasing population screening for osteoporosis. In assessing the risk of vertebral fracture, CT densitometry of the spine is equal or superior to DXA. This is justified by the fact that the analysis can only include trabecular bone, excluding cortical bone and vertebral posterior elements. In addition, CT values measured in Hounsfield units (HU) can be obtained prospectively or retrospectively from all clinical CT studies , and they can be used to estimate BMD with no additional costs or radiation (173). Within this subject Pickhardt et al. compared CT-attenuation values (in Hounsfield units) of trabecular bone between T12 and L5 vertebral levels with DXA BMD measures (reference standard). They found that CT-attenuation values were significantly lower in all vertebral levels for patients with DXA-defined osteoporosis ($P < 0.001$). Furthermore, they highlighted the results at L1 as these were more accurate than the results at other levels (174).

The clinical utility of bone mineral density that is CT-derived could be used for a fast identification of patients with a high-risk of fracture, addressing the problem by performing DXA; conversely it might exclude osteoporosis and make DXA unnecessary, thus reducing health costs and radiation.

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4.1.4 Sarcopenia

Over the last few decades there has been a widespread interest in research on sarcopenia, which is now formally recognized as a disease with an ICD-10-MC diagnosis code (175). According to the latest definition provided by the European Working Group On Sarcopenia in Older People 2 (EWGSOP-2), sarcopenia is a progressive and generalized skeletal muscle disorder and it is associated with an increasing likelihood of adverse outcomes such as falls, fractures, physical disability and mortality (176).

This definition has developed recently and muscle strength is now included in it, while the former definition was based on muscle mass only (177). According to the latest studies, muscle strength seems to be a more reliable parameter to predict the adverse outcomes mentioned above (178, 179). Moreover, alterations in muscle strength appear to be related not only to changes in muscle quantity but also to deep alterations in muscle quality, itself caused by the modifications in the architecture and composition of muscle cells (176). Through this evidence the latest guidelines identify muscle strength as the primary parameter to be evaluated in order to detect sarcopenia (176).

Considering the well-established negative impact of sarcopenia on several pathological conditions, every clinician in charge of patients with chronic diseases should be obliged to evaluate and exclude the presence of sarcopenia by using validated case-finding tools.

Among such tools the SARC-F questionnaire is the most used in daily clinical practice for patients aged ≥ 65 (180). It is a self-report questionnaire with low sensitivity but high specificity, based on the patient's self-evaluation of five motor abilities, i.e. walking, raising from a chair, climbing stairs, carrying weights and avoiding falls (180). Another recommended case-finding tool is the Ishii screening test, which considers age, hand grip strength and calf circumference (181). Nevertheless, the addition of an evaluation of skeletal muscle strength to the Ishii screening test would make it a more reliable tool in detecting sarcopenia.

If the screening tests come back positive, the evidence of low muscle quantity or low muscle quality should confirm a formal diagnosis of sarcopenia, through the use of tools available to this purpose. In clinical practice, tool selection may depend upon

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several variables which are related both to the patient and to the healthcare setting (176). A brief overview of these tools is presented in the Table 1 below.

Tab 6. *Validated tests for the assessment of muscle strength and muscle quantity.*
GS: grip strength; GST: grip strength test; CST: chair stand test; ASMI: appendicular skeletal muscle index; SMM: skeletal muscle mass; BIA: bioelectrical impedance analysis; DXA: Dual-energy X-ray absorptiometry; CT: computed tomography; MRI: magnetic resonance imaging; MCSA: muscle cross-sectional area.

Variable	***Parameter***	***Test***	***Tool***	***Advantages***	***Disadvantages***
Skeletal Muscle Strength	GS	GST	Dynamometer	• Simple and inexpensive.	• Provides only an approximation for strength of arm muscles. • Not possible to perform in case of hands disability.
		CST	None	• Simple and inexpensive.	• Provides only an approximation for strength of leg muscles. • Not possible to perform in case of legs disability.
Skeletal Muscle Quantity	SMM	ASMI	BIA; DXA	• Detailed information on the body composition • Relatively low-cost method. • Short time required.	• Trained physicians are required • Use of ionizing radiations (DXA)
	SMM	MCSA	CT; MRI	• The gold-standard methods.	• Highly trained personnel is required. • Expensive tests. • Time-consuming. • Use of ionizing radiations (CT). • Cut-off points for low muscle mass are not well defined yet.

Physical frailty is strongly related to sarcopenia, for this reason a comprehensive care plan for frailty should address the management of sarcopenia. Losses of skeletal muscle mass and muscle strength are often present in elderly subjects; such losses are independent predictors of adverse outcomes in trauma, cancer, chronic disease, and major surgery (182).

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Buchman et al., identified an independent association between mortality in patients with pneumonia and their respiratory muscle strength and extremity muscle strength, further connected to their pulmonary function (183). This research prompted a group of Italian radiologists to investigate the potential contribution of CT-derived muscle status to the prediction of clinical outcomes in COVID-19 patients, by using electronic density expressed in Hounsfield units and cross-sectional areas of the paravertebral skeletal muscle mass in axial CT images at T5 and T12 vertebral. They demonstrated that a combined model based on muscle status and lung disease is a good predictor of death in patients affected by SARS CoV 2 pneumonia (182).

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4.2 THE RESEARCH PROJECT 3

4.2.1 Introduction

Frailty is a condition characterized by a declining function across several homeostatic systems, leading to increased vulnerability to stressors and the risk of adverse health outcomes (1, 48). As is well known, frail older people have been the most affected during the COVID-19 pandemic. Several studies described the association of COVID-19 with frailty and mortality in patients aged 65 years or older (184-189). However, there still is a gap in the literature concerning pre-existing changes in organ and tissue, which may drive vulnerability and reduce resistance to stressors as acute as SARS CoV 2 pneumonia in the case of elderly people.

A recent international, multi-centred, retrospective observational cohort study took place across 63 hospitals in 11 countries in Europe and included 2434 patients. This study suggested that CFS score is a suitable risk marker for hospital mortality in adult patients with COVID-19 (167).

This consideration encourages the researcher to investigate body composition and in particular sarcopenia, not only as a marker of frailty in elderly people, but also as a predictor of poor prognosis in COVID pneumonia. Emerging data about SARS CoV 2 pneumonia revealed low muscle mass and visceral fat to be adverse outcome predictors in COVID 19 patients (182, 190).

As argued earlier arterial calcification, in particular thoracic aorta calcification, is another recognized prognostic indicator of biological ageing. Thomas et al. found that the calcium measurement of descending thoracic aorta was associated with non-CVD mortality, including COPD, hip fracture and pneumonia (172).

Furthermore, vascular calcification and osteoporosis follow the same pathway of pathogenesis. Animal models suggest the presence of abnormalities linked to ageing: for example mice with defects in the klotho gene expression show short lifespan, emphysema, osteoporosis and calcification of the medial layer of the aorta (90, 191).

In this study we wanted to identify changes in tissues that could be linked to frailty and vulnerability. We aimed at investigating retrospectively the behavior of skeletal

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muscle mass, thoracic aortic calcifications and bone mineral density using chest CT measurements.

Looking at the data that had been published prior to our study, we selected anatomical districts of thoracic high-resolution computed tomography to be studied:

- *Assessment of skeletal muscle mass (SMM)*: area (cm2) and density (HU) of paravertebral SMM at the Th12 level (182, 192).

Recently a relation between sarcopenia, diagnosed by calculating skeletal muscle area, and poor prognosis has been revealed in patients with various types of malignancies. Several studies showed that sarcopenia is an independent predictor of poor postoperative survival in patients with lung cancer (193). In addition, a correlation has been reported between skeletal muscle density and mortality in mechanically ventilated patients (194).

- *Descending thoracic aorta calcification (DTAC)*: DTAC was defined as the presence of calcium within the wall of the descending thoracic aorta visualised on CT, measured from the bifurcation of the pulmonary artery superiorly to the apex of the heart inferiorly. (171, 172).
- *BMD L1*: vertebral BMD by placing a single oval click-and-drag region of interest (ROI) over an area of vertebral body trabecular bone and then measuring CT attenuation in Hounsfield units (HU), with lower HU (lower attenuation) representing less-dense bone, at L1 level (174).

The BMD by CT at L1 is demonstrated to be more consistent than other vertebrae if compared to DXA measures for identifying osteoporosis. L1 vertebra is included on all standard chest and abdominal CT scans and this factor increases its potential screening use. It is easily identified and this improves efficiency and reproducibility. The accuracy of the method is independent from the presence or absence of intravenous contrast. Furthermore, the measurement can be applied retrospectively (174).

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4.2.2 Aim of the study

On the basis of the work reported above, we hypothesised that the complex pathophysiology of frailty in elderly people involves pathways that are common to muscle mass, macro vascular calcifications and bone density.

The purpose of this pilot study was to investigate on a single thoracic CT the relationship between paravertebral skeletal muscle area (cm2) and density (HU) at the Th12 level, descending thoracic aortic calcification (Agatston Score) and L1 bone mineral density (HU) in patients over 75 years of age affected by SARS-COV-2 Pneumonia.

4.2.3 Materials and methods

Study design and population

This retrospective observational study enrolled consecutive patients with SARS-CoV-2 infection who underwent thoracic HRCT following the physician's indication in the Emergency Department and who were admitted to the Internal Medicine Department 1 and Intensive Care Unit of S. Maria delle Croci Hospital in Ravenna. This study took place over 31 days of the second wave COVID-19 pandemic.

All patients presented positive RT-PCR tests in respiratory samples and a diagnosis of Pneumonia COVID-19-related (195).

Exclusion criteria were age < 75 years, absence of acute Coronavirus infection, poor quality of thoracic HRCT or absence of one or more anatomical districts selected for analysis. Patients who were hospitalised at the same time as the data collection were also excluded as the outcome of their hospitalisation was unknown (Fig. 20).

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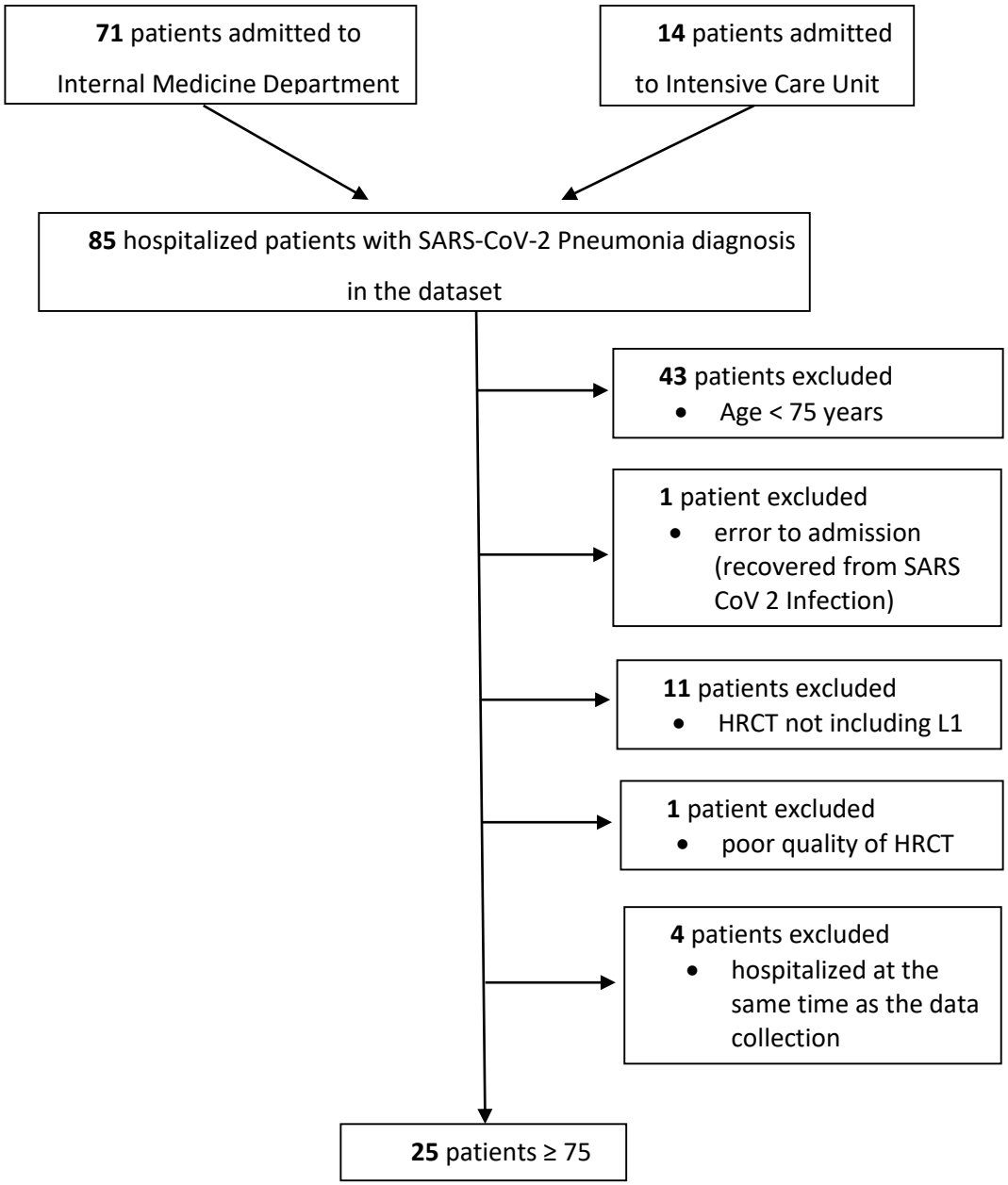
FIG 20: *Inclusion criteria flow chart.*



Study procedures and biochemistry

HRTC examinations had been performed at the Unit of Radiology in the Santa Maria delle Croci Hospital (Ravenna, Italy). High-quality images had been generated using a General Electric (GE) CT 128-slice system or a Philips CT 64-slice system at a resolution of 0.476562Å ~ 0.476562Å ~ 1 voxel size. The images were retrieved from the PACS archive and transferred to the Philips Intellispace Portal 9.0 console. The images underwent preliminary post-processing through the Multi Modality

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Advanced Vessels Analysis protocol and specific MPR reconstructions. The calcified atheromas of interest were manually selected. In particular, the study was extended to the aorta tract between an upper plane passing through the bifurcation of the pulmonary artery and a lower plane passing through the apex of the heart (172). The system automatically calculated the Agatson score by applying a threshold of 130 UH (196). In order to measure bone density, we drew a circular ROI with a diameter of 3 cm in the center of the vertebral body of L1. Finally, the measurements of paravertebral muscles were based on a single slice passing through the body of L1. In particular, the muscles' perimeter was drawn on both sides by using the function "ellipse" of the Multi Modality Viewer. Data was finally manually exported on an Excel data sheet.

Blood was collected from all participants on the first day of hospitalization for routine biochemical analysis. Routine biochemical blood tests included complete blood count, International Normalized Ratio (INR), D-Dimer, electrolytes, serum creatinine, high-sensitivity C-reactive protein, alanine amino transferase (ALT), alkaline phosphatase, lactic dehydrogenase (LDH), total bilirubin, vitamin D, phosphorus and parathyroid hormone.

The protocol was consistent with the principles of the Declaration of Helsinki.

4.2.4 Results

The choice of patients with age ≥ 75 years aimed at reducing the impact of age and at making the sample more homogeneous. The study included 25 patients with a mean age of 83±2.83 years, 12 of whom were females. The biochemical features of the population are showed in Table 7.

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Table 7 *Biochemical features of patients ≥ 75 years affected by SARS CoV 2 pneumonia.* *Continuous variables are presented as median [25th-75th percentile] as appropriate. Categorical variables are represented as percentages.*

	N = 25
Age, years	82.5 (5.2)
Male gender	52%
Leukocytes, 10^9/L	6.73 [5.37-8.33]
Neutrophil, 10^9/L	5.59 [4.45-6.59]
Lymphocytes, 10^9/L	0.65 [0.47-1.085]
PLT, 10^9/L	184 [134-270.5]
Haemoglobin, g/dl	12 [10.4-13.25]
INR	1.1 [1.05-1.24]
D-Dimer, ug/L	873 [639-1272]
Creatinine, mg/dl	1.14 [0.875-1.3]
Sodium, mMoli/L	136 [134.5-140.5]
Potassium, mMoli/L	3.8 [3.5-4]
Calcium, mg/dl	8.6 [8.4-8.7]
Magnesium, mg/dl	2 [1.7-2.17]
Phosphorus, mg/dl	3.11 [2.4-3.9]
Total bilirubin, mg/dl	0.48 [0.33-0.57]
ALT, U/L	21 [14-25]
Alkaline phosphatase, U/L	69 [62-87]
LDH, U/L	316.5 [255.25-373.5]
PCR, mg/L	80.9 [20.9-122.2]
25-OH Vitamin D, ug/L	24.09 [11.73-28.9]
PTH, ng/L	68.5 [59.75-106.25]

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For each individual the chosen radiological variables were analyzed on the first thoracic CT performed on admission to the hospital. The radiological features of the population are summarized in Table 8.

Table 8 *Radiological variables of patients ≥ 75 years affected by SARS CoV 2 pneumonia. Continuous variables are presented as median [25th-75th percentile]*

POPULATION	N = 25
AGATSTONE SCORE	3117.64 [1092.88-5127.33]
BMD L1, HU	75 [46-102]
SKELETAL MUSCLE AREA T12, mm² (sum right and left side)	2789.17 [2458.91-3202.1]
SKELETAL MUSCLE DENSITY T12, HU (average right and left side)	15.2 [5.25-27.3]

Agatstone score was inversely associated with L1 density (rho=- 0.452, p=0.024), with muscle density (rho=- 0.43, p=0.03) and with muscle area (rho=-0.54; p=0.005) Fig. 21. The relation found between Agatstone calcium score and L1 and muscle density remained significant after adjustment for possible confounders (age and sex), while the relation between Agatstone and muscle area disappeared after adjustment for sex.

No significant association between Agatstone Score and age was found (p=0.13). Furthermore, L1 density was directly associated with muscle mass density (rho=0.42, p=0.03) Fig. 22.

Moreover, the ROC curve was constructed to evaluate the discriminating power of the total DTAC, quantified according to the Agatston method, in order to predict a bad outcome (death) of patient affected by COVID pneumonia. The area under the curve (AUC) was 0.81. The cut-off value of DTAC = 2930,98 presented better values for sensitivity and specificity of 89% and 69%, respectively Fig. 23.

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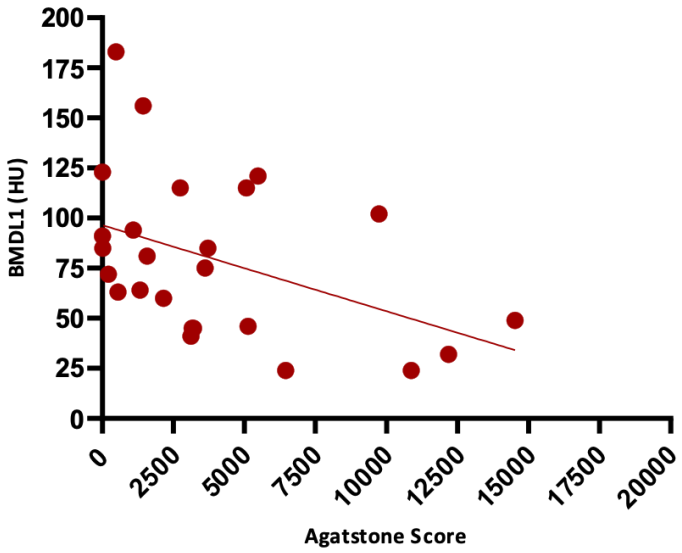
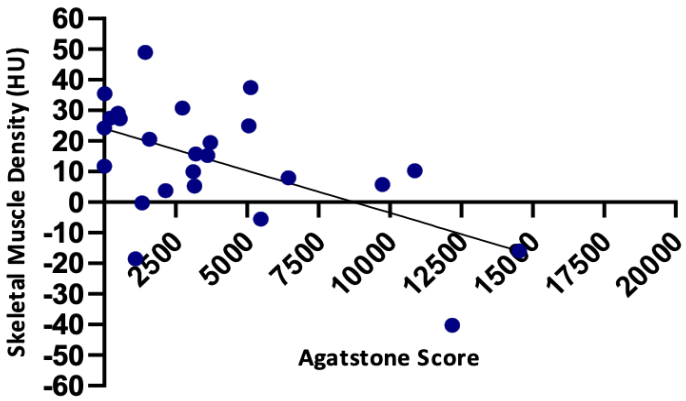
Fig. 21 *Correlation: a. Agatstone score vs ROI L1 (HU); b. Agatstone score vs Skeletal muscle density (HU); c. Agatstone score vs Skeletal muscle area (mm²).*

a.



b.



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c.

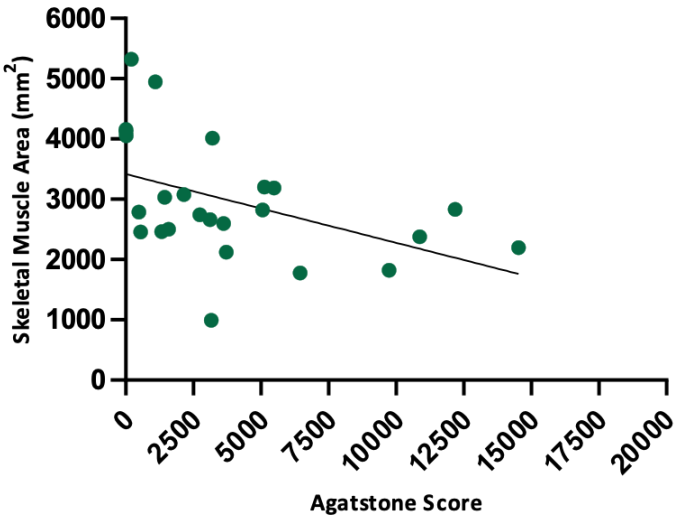
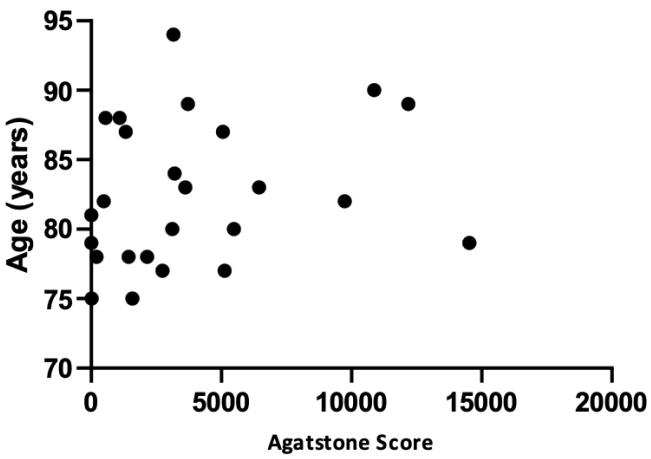


Fig. 22 *a. Scatterplot Agatstone score vs Age (years); b. Scatterplot ROI L1 (HU) vs Skeletal muscle density (HU).*

a.



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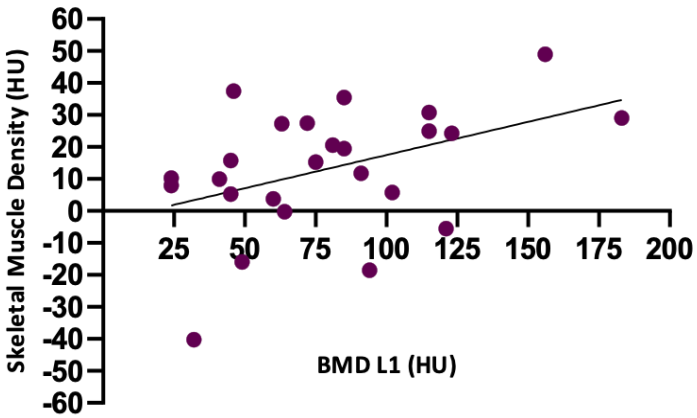
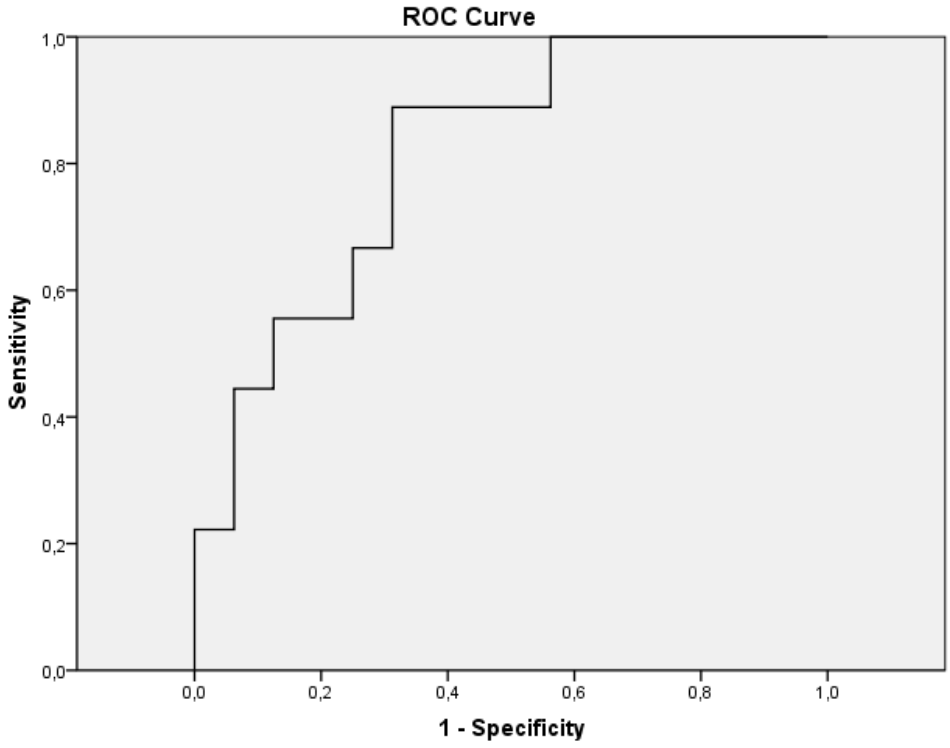


Fig. 23 *ROC Curve of DTAC (Agatstone score) in patients ≥ 75 years affected by SARS CoV 2 pneumonia.*



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4.2.5 Discussion

Through this study we found that calcium score of descending thoracic aorta has a good diagnostic power to identify an increased risk of death in patients aged 75 years or older who are affected by SARS CoV 2 pneumonia.

As far as we know, this study analysed for the first time three different tissues at the same time on the same radiological exam for each patient affected by COVID pneumonia. An inverse relation between thoracic aorta calcium score, bone trabecular density and skeletal muscle mass density was found, independently from age and sex. A significant and direct relation was found between L1 bone density and paravertebral muscle density.

In terms of the biochemical features of our populations, lymphopenia reflect the data from the literature of the last year and it has been defined as a reliable indicator of the severity and hospitalization in SARS CoV 2 patients (197). Contrary to the data reported so far (198) in elderly in particular (199), vitamin D status doesn't seem to affect the group of study. Analysing accurately this inconsistency, a possible explanation may be related to the absence of the value about bone metabolism and the small study sample. For the same reason the correlation between the vitamin D values and the Agatstone score is not satisfactory. Considering the only available data, 60% (6/10) of the patients presented vitamin D deficiency, defined for serum levels <20 ng/ml, and this prevalence data is in accord with the literature (199).

There is increasing evidence of a common pathway between vascular calcification and bone metabolism; coincidentally ageing is characterised by the development of osteoporosis and vascular disease. Several studies demonstrated an inverse association between bone mineral density and vascular calcification. In addition, high bone turnover is associated with increased CV mortality in elderly individuals, independent of age, gender, PTH serum levels and previous hip fractures (89). Bone demineralization and vascular mineralization represent a contradictory association that is commonly referred to as the bone-vascular axis. The results of our study confirm the presence of an inverse association between the calcium measurement of the descending thoracic aorta calculated through the Agatstone calcium score and the density of trabecular bone of L1 in patients over 75 years.

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Recently Giannini et al found that coronary aortic valve and thoracic aortic calcium assessment on non-gated CT allow to stratify COVID patients according to in-hospital mortality risk (200). In particular the total value of thoracic calcium – including the coronary artery calcium score, aortic valve and thoracic aorta calcium– is a stronger predictor of mortality than coronary artery calcium alone in COVID patients (200). Differently from the study just mentioned, we focused our investigation on the assessment of descending thoracic aorta calcium for two reasons. Firstly, as previously stated, it is demonstrated that TAC is able to identify individuals with increased vulnerability to non-CVD related mortality and non-CVD morbidity, especially chronic obstructive pulmonary disease, hip fracture and pneumonia (172). The second reason is given by the difference between medial and intimal calcification: Abramowitz et al. demonstrated the presence of both atherosclerotic and non-atherosclerotic process in the thoracic aorta calcification (201). Medial calcification, due to non-atherosclerotic pathway, may reflect biological ageing and it is commonly reported in aorta, not as frequently in coronary arteries. This can justify the discrepancy between TAC and CAC in the association with non-CV mortality. It becomes clear that, by selecting only the descending segment of thoracic aorta, we tried to reduce the impact of CV risk factors and the presence of anamnestic CV disease.

In elderly people bone loss often suggests the presence of osteoporosis, which is correctly defined as a systemic skeletal disease with consequent increase in bone fragility and susceptibility to fracture (137). Over the last decades, scientists focused their attention on the interaction between bone and muscle, not only because of the importance of this interaction for the musculoskeletal system, but also for the existing complex chemical and metabolical interactions (202). Similar risk factors, including genetics, endocrine and environmental factors, are recognized to be at the basis of sarcopenia and osteoporosis and both conditions are associated with ageing. The loss of mineral density in bone seems to be coincidental with decreased muscle mass, strength, and function and it is today accepted as a single disease named as osteosarcopenia (202). Clinically it is easily understood that osteosarcopenia is associated with increased risk of falls, fractures, frailty and mortality. Our results are in line with the literature, thus supporting a direct association between bone density

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and muscle mass. Our data specifically confirmed the muscle density, which reflects the amount of intramuscular fat content. Recent studies showed that muscle density correlates with muscle strength and this measurement could be important in order to diagnose and screen sarcopenia (203).

It is important to remember that sarcopenia and osteoporosis could be found simultaneously in a subset of the population, presenting a third entity called osteosarcopenic obesity (OSO) which has worse health outcomes compared to the individual disorders (204). Over the last year obesity and sarcopenia have been examined separately and both have been identified as risk factors of mortality in COVID infection. Considering the synchronous trend of bone loss and sarcopenia, in our study an inverse correlation emerged between DTA Agatstone calcium score in muscle density and muscle area measured at the level of T12. Nevertheless, after correction for sex the correlation with the area was lost.

In conclusion our study investigated the behavior of muscle mass, bone density and vascular calcification, which were examined on chest CT-scan in elderly people affected by COVID pneumonia. We demonstrated how vascular calcification is inversely related to bone mineral density and muscle mass density, while bone and muscle density are directly correlated. DTA Agatston calcium score in elderly with SARS CoV 2 pneumonia has a good power to discriminate the surviving patients from those who dead.

In the future a model with a single thoracic CT can be created to predict the overall outcome for elderly patients hospitalized for COVID Pneumonia. In addition, if the model will be validated, it could be applicable to other lung or oncological diseases. The model will be useful to identify the level of assistance needed for each patient, to improve the management of health resources and costs, to address patient with poor prognosis in palliative care, and finally to prevent and treat different biological markers of ageing and frailty.

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