# Is extravascular and intravascular calcification connected in patients with atherosclerosis?

PhD Thesis

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# **List of Abbreviations**

- ABI ankle brachial doppler index
- AHSG alfa-2 Heremans Schmid glycoprotein
- ALP alkaline phosphatase
- BAP bone specific alkaline phosphatase
- BGLAP osteocalcin
- bCTx beta-crosslaps
- BMD bone mineral density
- BMP bone morphogenetic protein
- BS Bollinger score
- CES cardio embolic stroke
- CS calcification score
- CI confidence interval
- CKD chronic kidney disease
- CRP C-reactive protein
- CVC calcifying vascular cell
- ECM extracellular matrix
- EDRF endothelial-derived relaxing factor
- ENPP1 ekto-nucleotide pyrophosphates -1
- DEXA: Dual-energy X-ray absorptiometry
- f-BMD: femoral head bone mineral density
- GFR glomerulus filtration rate
- HbA1c haemoglobin A1c
- HDL high density lipoprotein
- HR Hazard ratio
- IMT intima media thickness
- IQR interquartile range
- CS calcification score
- GP General Practitioner
- 1-BMD lumbar vertebral bone mineral density
- LDL low density lipoprotein

MGP - matrix glutamic acid protein

- MI- myocardial infarction
- MMP matrix metalloproteinase
- NPP1 nucleotide pyrophosphatase/phosphodiesterase
- NVE novell vascular event
- OPN osteopontin
- OR odds ratio
- ox-LDL oxidised LDL
- PAD peripheral artery disease
- PTH parathormon
- r-BMD: radial head bone mineral density
- ROC reciever operating characteristics
- ROS- reactive oxygen species
- SNP single-nucleotide polymorphism
- TGF- $\beta$  transforming growth factor- $\beta$
- $TNF\mathchar`-\alpha$  tumour necrosis factor- $\alpha$
- VEGF vascular endothelial growth factor
- VSMC vascular smooth muscle cell

#### Summary of investigations - about the findings that this study will present

The connection between intra and extravascular calcification has been evaluated. Initially, we determined the prevalence of osteoporosis amongst patients with lower limb atherosclerosis followed by the comparison of bone mineral density and some other parameters of osteoporosis to the severity of atherosclerosis. There are 3 main hypotheses explaining the connection between low bone mineral density and atherosclerosis in the normal population. The first one suggests reduced blood flow to the bones as a result of generalised atherosclerosis, the second dyslipidaemia and a third low vitamin D3 as responsible causes. All of the 3 parameters were noted and their affect on bone mineral density has been evaluated in our atherosclerotic patient group. In a different patient group, the association of complement components to the clinical parameters of chronic lower limb ischemia have been evaluated. In a follow up study we determined the effect of baseline complement component 3 and fetuin-A on future cardiovascular morbidity.

#### I. INTRODUCTION

Atherosclerosis and subsequent cardiovascular disease are a leading cause of death in the Hungarian population. The morbidity and mortality related to myocardial ischemic events have improved dramatically over the last few years in Hungary. Unfortunately, the outcomes for patients with peripheral arterial disease have not improved and indeed in some aspects have worsened [1]. Consequently, there is an urgent need for research into this field. Calcification is an often investigated process, and several recent findings suggest a connection between the vascular (ectopic) and the extravascular form. Furthermore, population based clinical studies described an association between atherosclerosis, osteoporosis and bone mineral density. Their effect on each other, however, is not clear. It is well known that beside the poor outcomes and low life expectancy, the quality of life of vascular patients is often impaired by severe comorbidities, such as diabetes, obesity or renal disease. The appropriate management of these comorbidities and to slow down the progression of atherosclerosis are essential in order to improve these patients' lives. Based on recent research of calcification, osteoporosis can be one of these so far less known comorbidities.

The understanding of atherosclerosis has much improved over the decades since the first population based study, the Framingham study, was launched. The identification and understanding of the risk factors of the disease is an ongoing process. The development of vascular calcification from the atheroma is a highly investigated mechanism and is one of the most clinically significant processes in the progression of the disease. Several markers, such as Complement components or fetuin-A have been associated with vascular disease and calcification. However, less is known about their role in the progression of atherosclerosis and the way they affect cardiovascular outcome. The purpose of this research is to investigate the connection between intraand extravascular calcification through bone mineral density and other calcifying parameters in patients with atherosclerosis.

# I.1. ATHEROSCLEROSIS

#### I.1.1. The pathophysiology of atherogenesis

#### I.1.1.1 Introduction

Many aspects in the development of atherosclerosis have been thoroughly investigated and fully understood. The existing knowledge of the disease however, expands and changes rapidly. Current research shows that the initial step in atherogenesis is endothelial injury and consequent dysfunction accompanied by extracellular lipid accumulation in the vessel wall [2]. The important role of mechanical factors in atherosclerosis has been highlighted back in 1957 [3, 4]. One of the most important risk factors, hypertension, is a clinical manifestation of increased shearing forces to the wall of the arteries [5, 6]. Many patients with essential hypertension have been diagnosed with endothelial dysfunction and it has been highlighted that these patients have an increased risk to developing further organ damage[7]. Endothelial dysfunction alters the permeability to certain molecules such as low density lipoprotein (LDL), results in vasoconstriction, leukocyte adhesion, thrombosis, and proliferation [8] and it could explain the higher incidence of atherosclerosis in patients with chronic inflammatory disease [9].

#### I.1.1.2 The role of lipoproteins

Following the injury of the innermost layer of the vessel wall the LDL settles in the sub endothelial layer. Endothelial damage will facilitate the aggregation of platelets which will synthetize vaso-active substances such as serotonin and histamine. The aggregated platelets will also induce smooth muscle proliferation [10]. These muscle cells will migrate towards the endothelial layer and secrete autocrine inflammatory proteins such as cytokines and interleukins [11]. The uptake of different molecular weight lipoproteins, especially the LDL, is regulated by the scavenger receptors[12]. Styrene based lipoproteins innate an inflammatory-immune reaction and macrophages will engulf and digest them and transform to foam cells [13]. These cells are dividing rapidly forming a lesion called fatty streak, which contains monocyte-derived macrophages, macrophage-derived foam cells, and T lymphocytes. These cells are all filled with lipids.

#### I.1.1.3 Inflammation

As atherosclerosis progresses, chronic inflammatory processes will mediate the process, which will eventually lead to the development of a complex lesion. The importance of the inflammatory cells has been demonstrated first on intercellular adhesion molecule (ICAM)-1 and P-selectin deficient mice. In these subjects virtually no atherosclerosis can be detected [14]. The raise in adhesional molecules such selectins, integrins and immunoglobulins will help inflammatory cells to attach to the activated endothelium [15, 16]. These cells will release reactive oxygen species (ROS) which will then oxidase LDL in the intimal layer and facilitate further lipid accumulation. Furthermore, it has also been described that inflammation itself increases the permeability of the vessel wall for lipoproteins [17]. The continuous retention of lipids will result in endoplasmic reticulum stress, hence, apoptosis [18]. A central region with increased inflammation and apoptosis will develop in the atherosclerotic plaque. Unlike acute inflammation where pro-inflammatory molecules are followed by anti-inflammatory ones, this chronic process will remain unresolved. This failed resolution is a new field of research for therapeutic agents [19]. The ongoing inflammation results in the vulnerability of the plaque accompanied by further apoptosis and smooth muscle cell death induced by inflammatory cytokines. This will eventually lead to fibrosis and formation of a fibrous cap [20].

#### I.1.1.4. Other important factors

In the growth of the fatty streak the matrix metalloproteinases (MMP) play an important role [21]. These enzymes are responsible for the degradation of extracellular matrix. However, they also play an important role in the smooth muscle proliferation and in the formation of neointima following endothelial injury [21, 22]. Furthermore, they are reported to be responsible for plaque instability [23]. They have many effects on

atherosclerosis, including regulating endothelial cell invasion, migration and lumen formation (angiogenesis) [24].

In response to tissue hypoxia, neovascularisation starts from the vasa vasorum of the adventitial layer towards the innermost intimal layer[25]. This is followed by increased angiogenesis promoting factors. The increased cell wall activity disrupts the homeostasis, leading to necrosis and inflammation. Consequently, this is associated with advanced atherosclerotic lesion and increased plaque vulnerability [26].

Vascular Endothelial Growth Factor (VEGF) affects both hypoxia and inflammation, and also induces neovascularisation in the vessel's wall. The administration of anti VEGF neutralising antibody in rats resulted in the stop in growth of coronary collateral circulation[27]. Further animal studies were able to demonstrate that the inhibition of VEGF receptors, for example by vaccination, was able to reduce the size of atherosclerotic plaques and the microvascular density [28, 29]. Some clinical data also confirms the important role of VEGF in atherosclerosis. Increased VEGF activity has been shown in relation to interplaque haemorrhage, a sign and cause of plaque instability in carotid endarterectomy specimens in symptomatic patients. The same activity could not be detected in patients without symptoms[30].

Additionally, abnormal vaso activity is shown in response to the injury and endothelia dysfunction. In healthy individuals the endothelial secretes Nitrogen oxide that acts as a vasodilatator. In atherosclerosis the secretion of NO is decreased resulting in vasoconstriction[31]. Furthermore, the bioavailability of NO is also decreased due to the excess of reactive oxygen species (ROS)[32]. This deteriorates to tissue hypoxia.

These processes over many years will result in a complicated lesion, which then will narrow the vessel lumen worsening tissue hypoxia and resulting in organ damage. The complicated lesion can transform to a vulnerable plaque, which is then most likely to be ruptured and result in acute thrombo-embolic event. Several attempts have been made recently to detect vulnerable plaques in order to prevent further clinical progression of the disease [33, 34].

#### I.1.2. Risk factors of atherosclerosis

#### I.1.2.1 Framingham Heart Study

Simultaneously to the studies on the pathophysiology of atherosclerosis, population based longitudinal prospective studies focused on identifying risk factors for atherosclerosis. Many years have passed since the first study, the Framingham Heart Study, was launched[35]. This was followed by many others focusing on the risk factors of atherosclerosis. The classic Framingham risk factors of atherosclerosis are: increasing age, hyper or dyslipidaemia, smoking, hypertension, diabetes, lack of physical activity, obesity, male gender and mental stress [35]. Over the years many other factors were investigated and some added to this list if they were found independently predictive.

#### I.1.2.2 New risk factors/markers of atherosclerosis

Several studies reported the connection between atherosclerosis and osteoporosis. Systemic vascular calcification is associated with low Bone Mineral Density [36]. Patients with osteoporosis are more likely to suffer from vascular calcification and peripheral vascular disease[37, 38]. Higher cardiovascular mortality also appears to be related to osteoporosis [39]. In our research, we thoroughly investigated this connection and we will present our findings below.

As described in the previous chapter, inflammation is a key element of the atherogenesis. Therefore, the effect of pro-inflammatory cytokines, immune molecules and acute phase proteins on atherosclerosis have been thoroughly investigated. Numerous studies described a strong association amongst C reactive protein and atherosclerosis [40]. However, the nature of this association is not fully understood. Recent randomised studies could not find a causative connection amongst them [41]. Beside CRP, the complement system [42], interleukins [43, 44], , tumour necrosis factor- $\alpha$  TNF-  $\alpha$  [45] and many more inflammatory agents have been identified playing a role in atherosclerosis. However, an ongoing issue is that the acute-phase proteins and

other inflammatory markers can only be considered as markers and not mediators of the disease [46].

Hyperhomocysteinaemia is one of the few risk factors that have been added to the classical risk factors of Framingham. Homocysteinylated lipoproteins with microorganisms obstruct the vasa vasorum and form vulnerable plaques[47]. It also induces endothelial dysfunction by damaging the endothelial cells[48].

The Human Genome Project made it possible to analyse some of the traits that lead to this multifactorial disease. Mainly single nucleotide polymorphisms have been identified as potential variants. The understanding of these genetic variants helps to better describe inflammation [49]. There is a long way to go in the research of the genetic background of atherosclerosis.

# I.1.3 The progression of atherosclerosis

The progression of atherosclerosis has a major effect on a patient's life. The risk factors for atherosclerosis and peripheral artery disease (PAD) are well known, however they cannot be used to assess the progression of the disease in symptomatic patients [50, 51]. Identifying the risk for rapid worsening of cardiovascular disease in patients with PAD and adjusting their therapy could help improve their quality of life, morbidity and mortality. Clinically, calcification appears to be a very important factor in the progression of the disease. The severity of calcification is strongly associated with cardiovascular mortality. Plaque fissures can be responsible for at least half of all cardiovascular morbidity, although morphologically, vulnerable plaques has a low positive predictive value for major cardiovascular events [52, 53]. The extent of calcification is linked to the instability of the plaques [54]. Therefore, there is a need to better judge the effect of extra and intravascular calcification parameters on the progression of the disease.

#### **I.2. VASCULAR CALCIFICATION**

Vascular calcification is an important feature of atherosclerosis as the degree of calcification is strongly associated to cardiovascular mortality. Most recent research suggested that -despite the historic view- vascular calcification is reversible [55]. This makes the understanding of the progression of calcification crucial to prevent the narrowing and obstruction of arteries at an early stage.

### I.2.1. Anatomical variation of calcification

Historically, cardio-vascular calcification has been treated as the same disease, however it is fully understood now that the manifestation of it at different anatomical sites develop and behave differently. Calcification most commonly affect elastic type arteries in the systemic circulation. The subtype of this concludes intimal or tunica media calcification and porcelain aorta, which is limited to the ascending part of this vessel. Pulmonary arteries are less likely to be affected by calcification and are usually associated with pulmonary hypertension. Arterioles can also be affected by calcification, usually in chronic kidney disease (CKD). This type of calcification is referred to as calciphylaxis. Cardiac valves, predominantly the aortic valve, can also calcify. This type of calcification shows many similarities to arterial calcification, however at the present time, it is considered a different disease. Finally, myocardial calcification has also been described. The pathogenesis of this disease is currently poorly understood[56]. These anatomical variations are further demonstrated in Figure 1.



Figure 1. The different types of calcification. Figure by Bostrom et al. [56] "Schematic drawing of different types of vascular calcification affecting elastic arteries in the systemic circulation, including atherosclerotic lesion calcification, calcification of the internal elastic lamina (IEL), coral reef aorta, media sclerosis (Mönckeberg's disease), and porcelain aorta".

# I.2.2. Development of calcification

# I.2.2.1. Introduction

According to Murshed et al. [57] the development of tissue bone mineralization requires two conditions: a fibrillar collagen rich matrix as a scaffold, and the expression of alkaline phosphatase. These two factors could be found in almost all tissue, including vessels. Different types of calcification have been identified in the adventitial and in the tunica media layers. The deposition of lipoproteins seems to be playing an important role in adventitial calcification but not in medial calcification [58]. The changes in the medial layer, also described as Möckenberg sclerosis, are associated with age, diabetes

and chronic kidney disease. This type of calcification is also visible on plain radiographs[59].

#### I.2.2.2. Vascular smooth muscle cells

Both layers, but especially the tunica media, are built up from collagen rich matrix and contain vascular smooth muscle cells (VSMC). These cells do not express alkaline phosphatase under healthy conditions, whilst under calcifying conditions alkaline phosphatase activity was measured [60]. Their migration is stimulated by VEGF molecules [61]. The importance of these VSM cells is the potential to transfer to osteoblasts. This could be facilitated by several factors. Intimal calcification appears to be mediated beside oxidative stress by bone morphogenic proteins (BMP) and the level of pyrophosphates. BMPs are inducing inflammation in the plaque facilitating intimal calcification [62].Several types of BMPs appear to be affecting calcification, but BMP-2 appears to be having the most important role as its level is increased in calcified plaques [63].

#### I.2.2.3. Vitamin D

Vitamin D plays a controversial role in vascular calcification and its effect is most likely be dose dependent [64]. Vitamin D excess leads to calcification in subjects with renal disease. At the same time its level inversely correlates to coronary calcification [65]. Vitamin D receptors have been identified in many cells including VSMC. A high dose of vitamin D induces osteoblastic phenotype of VSMC, and therefore, calcification. The effect of it is so strong that it has been used to induce calcification in animal experiments [66]. Klotho is a gene responsible for premature ageing and it inhibits vascular calcification in mice. The loss of this gene leads to ectopic calcification, but this can be reduced by genetically inhibiting the production of the active form of Vitamin D 1,25(OH)<sub>2</sub>D at the same time [67]. There are several ongoing clinical studies with Vitamin D supplementation in cardiovascular disease but the benefits of the routine use of Vitamin D have not been confirmed yet [68].

#### I.2.2.4. Further pathophysiology of calcification

Oxidative stress in relation with inflammation may also induce the process. The mechanism is not completely understood, but atherosclerosis is an inflammatory disease and vascular calcification is a big part of it. Additionally, many inflammatory cytokines have been identified being pro-calcific[69].

The regulation of phosphate levels is also an important feature in calcification. Nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) is responsible for the production of extracellular inorganic phosphate. The mutation of the NPP1 gene leads to general arterial calcification of infancy [70]. Renal failure leads to hyperphsophateamia, which consequently triggers vascular calcification [71] Matrix Gla proteins (MGP) have an important role in the inhibition of vascular mineralisation [72]. They affect the pathway in two different ways. They can directly block sclerotic crystal growth by interacting to hydroxyapatite and calcium ions [73] and they also regulate BMP-2 expression [74]. Furthermore, in animal experiments it has been shown that if this pathway is inhibited (in MGP -/- mice) the extent of calcification is less severe [75]

#### I.2.3. The role of complements in progression of calcification

The Complement System is a part of the innate immune system and it activates in 3 main pathways: the classical, alternative and the mannose binding lectin pathway. The activation of the classical pathway requires Complement 3 (C3) while the activation of lectin pathway requires Complement 4 (C4). Inflammatory mediators, such as C-reactive protein (CRP) or the components of complement are present in all stages of atherosclerosis[76]. The third component of complement (C3) is strongly associated to the severity of atherosclerosis [77, 78] and to cardiovascular risk factors in elderly individuals [79] (Please see Figure 2.). C3 increase vascular stiffness by binding to collagen and elastin fibres in the vessel wall [80]. During premature enchondreal bone formation increased activity of C3 was found[81] and its role in the post traumatic cartilage healing[82] was also verified. In female patients the level of C3 is associated

to the progression of plaques and intimal media thickness (IMT) [83], and also to arterial stiffness[84].

Furthermore, recent evidence suggests that the complement cascade is activated within the atherosclerotic plaque and they may play a role in plaque destabilization [85]. It has also been described that inflammatory changes precede plaque instability [86]. In patients with carotid artery disease, radiological unstable plaques had very low prediction for post interventional embolization, whilst inflammatory status was found to be a predictor of this [87]. Hence, inflammatory agents including C3, seem to be a possible factor for novel vascular events.

Clinical data also suggests an important role for complements in the progression of atherosclerosis. In healthy subjects non independent association was found between higher level of C3 and myocardial infarction[88]. Higher C3 level have been associated with cardiovascular risk in patients with psoriasis[89] and has been identified as a risk for coronary artery calcification in women with systemic lupus erythematosus [90].

This evidence suggests a connection between vascular calcification, bone formation and the serum level of third part and fourth part of complements.

I.2.4 The role of Fetuin-A in the progression of calcification

Fetuin-A also known as Hereman Schmidt glycoprotein is an important factor in the inhibition of calcification, thus on the overall progress of atherosclerotic disease. In the last decade, researches have focused more on regulatory factors associated to the severity of vascular calcification (Please see Figure 2.). These researches first focused on patients with chronic kidney disease (CKD). Among these patients the serum level of Fetuin-A[91] and pyrophosphates[92] are inversely correlated with the severity of calcification. Recent studies focused on patients without renal disease. These studies suggest that Fetuin-A has an effect on calcification among diabetic and non-diabetic patients with peripheral artery disease[93, 94], coronary disease[95] or aortic aneurism[96]. Additionally, they suggest that Fetuin-A has a major role in the inhibition of calcification.

# I.2.5 Other factors contributing to plaque development

Several other factors have been highlighted as potential markers of the progression of atherosclerosis. For example, VSMC cells have been identified as contributors to plaque instability[97] or arterial stiffness could also be a prognostic factor for cardiovascular mortality in patients with PAD[98]. VEGF over expression also has an important role in the progression of the disease by inducing plaque instability [30]. Local increase of elastase and consequent decrease in arterial stiffness is related to the production of elastin-derived peptides (EDP). The elimination of the effect of EDP appears to be a promising therapy, however further research is required [99]. In our study, we focused primarily on potential factors contributing to the calcification and development of the plaques such as complement protein or marker related to calcification (Please see Figure 2.).



Figure 2. Summary of factors influencing vascular calcification. Original Figure by Giachelli et al.[55]

#### **I.3. BONE FORMATION AND OSTEOPOROSIS**

#### I.3.1. Osteogenesis

Bone is derived from para axial mesoderm. There are 2 types of bone formation. Enchondreal ossification begins with formation of chondrocytes from mesenchymal cells and subsequent cartilogenesis. This is followed by the transformation of the cartilage to bone. The importance of this type of ossification is in the growth in length of the bones. Intramembranous bone formation is initiated from the neural crest originated mesenchymal cells. These cells develop as compound nodules and differentiate in to different cells such as capillaries or osteoblasts committed to bone formation. The most important role is in the development of the bones of the cranium and its role in bone healing [100].

#### I.3.2. Osteoporosis

#### I.3.2.1 Osteoporosis at a glance

The human bone is a constantly changing tissue. By aging, some of our bone cells dissolve, however simultaneously new bone forms. The remodelling of the bone this way has been first described in 1963 [101]. Up to 10% of the whole bone mass can undergo remodelling at a time. The responsible cells for bone degradation are the osteoclasts, whilst the osteoblasts are rebuilding the bone matrix. If the difference of bone formation and resorption turns negative and more bone dissolves than new forms, the bone mass starts to decrease. This leads to lower bone density and an increased risk of fractures [102]. Approximately 15% of the Caucasian population is affected by osteoporosis around the age of 50 and about 70% over the age of 80. It is more common in women than men [103]. The diagnosis of osteoporosis is based on the measurement of Bone Mineral Density, usually by Dual Energy X-Ray Absorptiometry [104].

#### I.3.2.2 Risk stratification and markers in osteoporosis

There are several factors highlighted to have an important role in the evaluation of osteoporosis [105]. The most important is to estimate and eventually decrease the risk of osteoporotic fractures. The suggested tool for the assessment of fracture risk by the WHO is the FRAX tool [106]. This calculation encounters the most important risk factors and BMD. These are age, sex, BMI, previous fracture, parent fractured hip, current smoking, glucocorticoids, rheumatoid arthritis, more than 3 units of alcohol per day and secondary osteoporosis (insulin-dependent diabetes, osteogenesis imperfecta in adults, untreated long-standing hyperthyroidism, hypogonadism, premature menopause, chronic malnutrition, and chronic liver disease). In addition to this, neuromuscular disorders, long-term immobilization and low dietary calcium intake have also be identified as potential risk factors[107]. The biochemical markers can be divided in to two subgroups based on bone absorption or formation. The most important markers of bone formation are total and bone specific alkaline phosphatase and osteocalcin. Resorption is best reflected in B crosslinks and hydroxiplorin. Parathyroid hormone and Vitamin D3 also reflects on bone homeostasis. Some of these risk factors are common for atherosclerosis, vascular calcification and osteoporosis.

# **I.4. THE CONNECTION BETWEEN VASCULAR CALCIFICATION, BONE FORMATION AND OSTEOPOROSIS**

#### I.4.1. History

Atherosclerosis has been identified as a calcifying disease in 1983. However, the first studies investigating the connection between osteoporosis and vascular calcification were published 10 years later, at the same time when atherosclerosis was suggested to be an inflammatory disease. The ongoing Framingham Heart Study suggested a connection between metacarpal relative cortical area and abdominal aortic calcification suggesting a connection between bone density and vascular calcification [108]. The classical definition of Calcific diseases describes that Ca uptake is increased,

calcification is significantly related to dysfunction, and the control of calcification may improve the outcome of the disease [109].

#### I.4.2 Bone cells in the arterial wall

O'Brien et al.[110] were able to demonstrate that vascular calcification is not a result of a degenerative process in the atherosclerotic plaque, but rather an actively regulated process. They were able to isolate osteopontin, a bone matrix protein from cardiac valvular and vascular calcification. This protein was found around calcium deposits and adjacent macrophages. They proved by in situ hybridisation that the osteopontin is secreted by these macrophages, but not by distant ones. Osteopontin is mainly secreted by osteoclasts and to some degree, by osteoblasts. Osteoblasts are single nucleated cells responsible for the synthesis of bone. Osteoclasts are multinucleated cells that develop from the same precursors as macrophages. Their importance is in the absorption of the bone. Under normal circumstances, their importance is in forming the bone marrow canal. The high number of osteoclasts leads to osteoporosis while low number results in osteopetrosis. Further evaluation of calcification demonstrated that the development of vascular calcification shows similarities to bone formation[111]. Several more bone matrix protein have been isolated from vascular calcification[112] suggesting ongoing chondrogenesis and osteogenesis.

As described earlier, vascular calcification can occur in several distinct layers of the vessel wall and they develop in alternate ways. Different types of bone formation have been identified in the intimal layer of the arteries and in the tunica media. Intimal calcification follows the sequence of enchondreal ossification while Möckenberg sclerosis resemble to intramembranous bone formation. The presence of bone morphogenic proteins (BMP), however, is not sufficient to describe the process and understand the link between ectopic and physiological calcification – bone formation. The question also remains as to where do osteoblasts derive from in ectopic places such as the vessel wall. The most likely explanation is that calcifying mesenchymal vascular cells are able to differentiate in to osteoblasts [113], but it has also been suggested that circulating mesenchymal precursors could be a source of them [114]. Another theory suggests migration of adventitial myofibroblasts, which then mineralises the cells[115].

Either way, vascular smooth muscle cells (VSMC) seem to be playing the most important role in the mineralisation and ossification of the vessel wall[116].

#### I.4.2.1. Aids of calcification

It has been shown that high calcium and phosphate level induces the differentiation of VSMC to osteoblasts facilitating calcification [117].

Angiogenesis is an important feature of cardio vascular calcification. It has been shown that Vascular Endothelial Growth Factor (VEGF) is a key regulator of vessel and bone formation during enchondreal osteogenesis [118]. It has also been demonstrated that it affects intramembranous bone formation and increases bone mineralisation [119].

#### I.4.2.2. Inhibition of calcification

The inhibition of ectopic calcification can occur through alkaline phosphatase binding with inorganic pyrophosphate. Pyrophosphate affects hydroxyapatite, which is a potent inhibitor of the development of calcium deposits in the extra cellular matrix. The level of pyrophosphate is controlled by ekto-nucleotide pyrophosphate phosphodiesterase (ENPP1). The lack of this gene will induce arterial calcification in children and severe calcification has been found in ENPP1 mice [120]. Parathyroid hormone (PTH) plays an important role in bone and calcium homeostasis. It has been shown PTH is able to reduce the calcification on VSCMs by inhibiting ALP in the extracellular matrix [121].

# I.4.3. Clinical associations

The molecular pathways of ossification-calcification in the vessel wall has been thoroughly studied and many aspects have been clearly identified. However, less has been found about the clinical relation of the different types of calcification. Cross sectional studies and population based research demonstrated the clinical association between the prevalence [37] and the severity [36] of the two diseases. Osteoporosis in patients with vascular calcification worsens the outcome of vascular diseases [39]. Comparison of patients suffering of osteoporosis with or without vascular calcification demonstrated that the outcome of osteoporosis is worse and the number of fractures are higher in the atherosclerotic group. Ankle brachial index (ABI) is a measure of the severity of atherosclerosis. It has been shown that the ABI is associated to bone mineral density (BMD) [122]. These associations represent a strong connection between these conditions but the exact origin of this relation remains unknown.

I.4.4. About the theories explaining the connection

I.4.4.1. Reduced blood flow – less nutrition

Many hypotheses have been proposed over the years to answer the questions about the origin of the connection between bone disease and vascular calcification.

Atherosclerosis and consequent calcification often affect the abdominal aorta and the ilio-femoral arteries. These vessels are important blood supply of the lumbar spine and the femoral head respectively. These bones are the most common sites of BMD measurements beside the radial heads. The reduced blood flow in these bones result in insufficient nutrition impaired bone repair mechanisms[37]. Some authors were reporting site specific association and suggesting that osteoporosis is a result of reduced blood flow due to vascular calcification [123]. A post mortem angiography based study demonstrated the blood supply of the lumbar spine, with the lumbar and medial sacral arteries being more likely to be occluded on angiography in subjects with a history of chronic back pain. This study also precisely describes the blood supply of the lumbar vertebras [124].

#### I.4.4.2. Dyslipidaemia

Another possible explanation for the described relation suggests an important role for dyslipidaemia in both type of calcification. Serum cholesterol level is an important risk factor of atherosclerosis [35]. Laboratory studies suggested that HMG-co reductase inhibitors, also known as "statins", are able to improve bone mass and slow down bone turnover. In vitro animal studies suggested that this could be achieved by increasing the production of BMP-2 [125]. The importance of BMP in calcification has been

previously explained. Furthermore, simvastatin was found to have a positive effect on BMD in postmenopausal patients with high cholesterol [126]. However, this association was not confirmed in later studies [127]. Overall, the role of dyslipidaemia in osteoporosis is controversial [128].

# I.4.4.3. Vitamin D

As described above, Vitamin D3 appears to be having an important role in vascular calcification and its positive effect on BMD is well known [129]. Many recent data suggest that vitamin D can be the link between the 2 diseases. Vitamin D receptors are located in many different tissue including endothelial cells and smooth muscle cells in the vessels wall. These cells produce the active form, dihydroxycholecalciferol in the kidneys. Vitamin – D deficiency has been associated in clinical and experimental studies with several worsening effects on vascular and extra vascular calcification [130]. Cardiovascular mortality [131], low BMD and increased osteoporotic fractures has all been linked to low vitamin-D levels.

### II. AIMS

II.1. Osteoporosis and atherosclerosis - Prevalence, connection, prognosis

According to our knowledge the prevalence, the severity and the treatment of osteoporosis has never been evaluated in the Hungarian population of patients with peripheral vascular disease. It is not known how many of these patients are affected by osteoporosis or how many of them have already been diagnosed and treated. The evaluation of BMD in relation to atherosclerosis is also a remaining question. This is of high importance in order to improve the outcome and the quality of life of these patients. To initiate treatment for osteoporosis appears to be necessary as ectopic calcification negatively influences bone mineralisation [132]. There is a need to further analyse the means of their connection too. The understanding of the nature of this can further aid novel therapies.

We evaluated the extent of osteoporosis and osteopenia based on huge variety biochemical markers (vitamin D3, PTH-, osteocalcin- (BGLAP-), bone specific alkaline phosphatase (BAP). beta-crosslaps- (bCTx-), and bone mineral density (BMD) in patients with severe chronic lower limb ischemia. We recruited patients with symptoms of lower limb ischemia in order to compare site specific bone density (lumbar, femoral and radial) to the site of the vascular lesion (aorto-iliac, femoro-distal). The risk factors for both diseases have also been noted.

II.2. The relation of Complement complements to the clinical parameters of lower limb atherosclerosis

It is known that the level of Complement 3 and 4 is associated with atherosclerosis and vascular calcification, however its relation to the clinical severity of atherosclerosis in patients with lower limb ischemia remains unknown [77]. The progression of atherosclerosis has a major effect on a patient's life. The risk factors for atherosclerosis and peripheral artery disease are well known, however they cannot be used to assess the progression of the disease in symptomatic patients [50, 51].

We evaluated the connection of C3 and C4 to the severity of atherosclerosis and vascular calcification by using a wild variety of methods.

II.3. The role of complement component 3 and Fetuin-A in the progression of lower limb ischemia

Complements have been associated with the worsening of atherosclerosis and vascular calcification. The role of fetuin-A in calcification has been previously described by our research group, however its role in the progression of the disease has not been published yet. Baseline C3 and Fetuin-A levels in a follow up study have been compared to middle term novel cardiovascular events such as stroke, myocardial infarction and further vascular operative intervention.

# **III. METHODS**

Consecutive patients with peripheral artery disease have been recruited at the Outpatient Department of Semmelweis University Department of Vascular Surgery in 2009 for the purpose of this study. Our inclusion criteria were: patients with present symptoms of atherosclerotic chronic lower limb ischemia or carotid disease who gave written informed consent. We excluded all patients with acute onset of ischemia, clinical or laboratory signs of acute infection, malignant tumour, hepatic disease, end stage renal disease (dialysis), immune suppression, severe medical or surgical conditions (myocardial infarction, stroke, trauma, surgical procedure) in the last 6 months. Patients with serum creatinine level > 100  $\mu$ mol/l or estimated glomerular filtration (eGFR) < 60 ml/min were also excluded from the study. Please find a summary of the the inclusion and exclusion criteria and the number of patients recruited to each study in Table 1.

	osteoporosis and	the association of	baseline C3 and
	vascular	complement	cardiovascular
	calcification	components and	outcome
		clinical parameters	
		of atherosclerosis	
number of patients	172	103	246
location of artery	symptomatic lower	confined to lower	peripheral artery
disease	limb disease	limb only	disease
inclusion criteria	chronic lower limb	chronic lower limb	chronic lower limb
	ischemia	ischemia only	ischemia or carotid
			disease
exclusion criteria	acute infection or ischemia, malignancy, liver failure, kidney		
	disease, immun suppression		

Table 1. Summary of different study groups, their inclusion and exclusion criteria.

#### III.1. Clinical evaluation

The initial study questioner is presented in original language Hungarian and English translation in brackets in Table 1. Age, sex and past medical history have been noted on the day of presentation to the outpatient clinic or on the day of consequent hospital admission. Past medical history focused on diabetes, ischemic heart disease, cerebrovascular event, liver and kidney disease as well as metal work or other device previously implanted in the patient. Body mass index (BMI) was calculated as weight (kg) / height<sup>2</sup> (m). Metabolic syndrome was identified by the presence of three or more risk factors (abdominal obesity, high triglycerides and high density lipoprotein (HDL), elevated blood pressure or treated hypertension, history of diabetes or elevated fasting blood sugar defined by the guidelines of the International Diabetes Federation [133]. Past and present smoking habits and alcohol consumption has been recorded. Furthermore, history of osteoporosis or osteopenia and treatment received has been noted. Current medical therapy, especially statins, anticoagulants/ anti thrombocyte aggregating agents were also asked in the study questionnaire. Our subjects were asked about their exercise tolerance and their walking distance. The traditional Fontaine classification was used to assess the clinical severity of the chronic lower extremity atherosclerotic disease (groups I, II/a, II/b, III, IV). Group II was separated to "a" and "b" subgroups at a walking distance of 200 meters.

Table 2: Patient Ques	stioner in origina	al language	Hungarian	and	English	translation
in brackets						

Beteg	adatlap	(Patient	Vizsgálati azonosító: (ID number)				
Questionna	ire)						
Demográfia (demographics)			Status				
Név (name)			Carotis stenosis (tünet, %, oldal, sebesség)				
TAJ (health insurance number)			(degree of carotid stenosis, flow,				
Születési i	dő (Date of Birt	h)	lateralisation)				
Cím (addr	ress)		Alsó végtagi panaszok				
Telefon (p	hone number)		(lower limb symptoms)				
Adatlap ki	itöltésének ideje	(time of entry	Dysbasiás távolság (walking distance)				
to the stud	ly)		Fontaine beosztás (Fontaine stadium)				
Súly (weig	ght)		Pulzus status (peripheral pulse status)				
Magasság (height)			Doppler boka-kar index (ABI)				
Haskörfogat (abdominal girth)			Aneurizma (Marfan) (aneurysm)				
Anamnézis (Medical History)							
Diabétesz I-II (diabetes)			Májbetegség (liver disease)				
Menopauza (menopause)			Uraemia/dialízis (kidney disease)				
Dohányzás (smoking)			Infekció (akut/krónikus) (current infection				
Osteoporosis			status)				
Hipertónia	a (hypertension)		Malignus betegség (malignant disease)				
ISZB/AM	I (IHD)		Immunszuppresszió (immunosuppression)				
Stroke			Idegen test jelenléte (foreign body)				
			Gyógyszerek (medication)				

#### III.2. Assessment of atherosclerosis and calcification

The methods used for the assessment of calcification have been previously described by our research group on several places [78, 94]. For the purpose of the thesis we provide a brief summary of the methods please read the above cited articles for full details. An experienced vascular surgeon performed physical examination and ankle-brachial index (ABI) measurement with Doppler ultrasound probe. The patients laid in a supine position after resting for pressure measurements over the posterior tibial and dorsal pedal arteries. ABI was calculated as the lowest pressure of the ankles divided by the higher of the left and right arm pressures[134, 135].

#### III.2.1. Imaging modalities

The extent of calcification was assessed by evaluating the carotid intima media thickness (IMT) and a General Calcification Score which were determined by a single experienced radiologist who was blind to the patients' clinical information. IMT was measured on a plaque free area at three points of the dorsal wall of the common carotid arteries, using a linear (7.5-11MHz) and convex (3.5-5MHz) transducer of a Toshiba Aplio SSA-770 ultrasound system. The mean value and the maximum IMT was used for calculations[136]. During the same examination carotid stenosis was also determined. Please also see Figure 3. To assess the overall extent of systemic atherosclerosis a calcification score (CS) was calculated after examining the vascular system at seven sites: both carotid bifurcations, the infrarenal aorta, both common femoral arteries, aortic and mitral valves by B-mode ultrasound (see technical details above at carotid IMT measurements). If calcification was noted, the spot was rated as 1. Sites with no calcification received 0. As we evaluated calcification at 7 sites the calcification range was 0-7 [137-139]. Transthoracic echocardiograms were performed by one experienced cardiologist blind to other study information. Examinations were performed, including Doppler images in all standard views using phased array transducers (2.5-4.5MHz). Mitral and aortic valve calcification was determined if echodense structures were noted at the appropriate views [140].



Figure 3. Assessment of carotid calcification by ultrasound for calcification score (investigations performed by Dr Endre Rimely at the Hear and Vascular Centre of Semmelweis University)

# III.2.2. Laboratory measurements

Blood samples were collected after a minimum 6 hours of starvation. These samples were used to evaluate laboratory characteristics of our study cohort. Conventional standardized methods were performed in the core laboratory of Semmelweis University. On admission Urea and Electrolyte, Full Blood Count, Clothing and Liver Function Test, C-Reactive Protein, HemogblobinA1c, Protein C, Lipid Profile were measured. We used the Cockcroft-Gault formula for the calculation of glomerular filtration rate.
III.3. The evaluation of osteoporosis amongst patient with Peripheral Artery Disease

### **III.3.1** Biochemical parameters

According to our knowledge, the prevalence of osteoporosis and osteopenia has never been investigated in the Hungarian population of patients with clinically manifest vascular disease. For the purpose of this part of the study, we investigated the BMD of our 172 patients with PAD. In addition to the baseline laboratory measurements we evaluated the level of vitamin D3, beta crosslaps (bCTx), bone alkaline phosphatase (BAP), osteocalcin (BGLAP) and parathyroid hormone (PTH). For the purposes of investigations regarding the role of Vitamin-D we divided our study cohort into high and low Vitamin-D subgroups. According to Holick [141], low level of Vitamin-D was noted if the patient had 20 mg/mL or lower serum concentration. Dyslipidaemia has been diagnosed for the purpose of clarifying its role in the connection of the diseases. For this purpose, either previous diagnosis of dyslipidaemia or according to Nataraja et al. [142] total cholesterol/ high Density Lipoprotein ratio has been used.

### III.3.2 Dual-energy X-ray absorptiometry

The recommended method by the WHO for the assessment of bone health is Dualenergy X-ray absorptiometry (DEXA) scan. The principal of the scan is based on the simultaneous use of two X-ray beams with different energy. By subtracting the soft tissue absorption from the images the difference between the penetration of the beams to the bone will determine the bone mineral density. The definition of BMD consists of the mineral content of a defined area of the bone surface. Please see also Figure 4. The BMD is measured on 3 different bones. The femoral (f-BMD) and radial (r-BMD) head, and the lumbar spine (l-BMD). The T-score has been calculated based on the different density to healthy bone. According to the WHO guidelines, patients with more than -2.5 SD to their age matched peak bone density were treated as osteoporotic. If the SD of the T-score was between -1 and -2.5 these patients were considered as having decreased BMD, hence osteopenia[143].



Figure 4. An example of a DEXA scan demonstrating how calcification appear and what type of results does the scan provide. (source: www.orthospinelab.com [144] )

# III.3.3. Angiography, Bollinger score

These methods have previously been described in Fehérvári et al [145].

Lower limb angiography was taken by using standard methods. The original pictures were analysed by an experienced radiologist, who was blind to other parts of the study. According to the clinical symptoms and to the angiographic images the site of the atherosclerotic lesion was also noted.

Angiography based scoring systems are not the most popular way of classifying atherosclerosis, mostly due their complexity. However, for the purpose of this study we have chosen an angiography based score system created by Bollinger et al. [23], also called the Bollinger score (BS). This allowed us to precisely demonstrate the lack of perfusion in the exact anatomical region. Two experienced radiologists -who were blind

from each other's results- analysed the pictures. An additive score was calculated in order to assess the extent of arteriosclerosis of the infrarenal aorta, iliac, femoral, popliteal and crural arteries on each side. Stenotic lesions and occlusions were noted in each arterial segment on both sides. Four categories of occlusive lesions were defined in descending order of severity: complete occlusion, stenosis narrowing the lumen by more than 50 %, between 25 and 50% and less than 25% (Lower score values were assigned to less severe stenosis.) If the stenotic area exceeded more than half of the length of the vessel, higher values were given and occlusion received the highest scores, especially if it was observed in the full length of the artery.

This score system is particularly suitable to assess systemic atherosclerosis, because it is able to judge stenoses and occlusions in a long segment of the vascular system. It is also particular suited for this study as it takes the anatomical segment in to account. The site of the atherosclerotic lesion was noted and compared to the BMD measurements, which were also site specific.

#### III.3.4. Site specific assessment

To enable site specific comparison amongst the vascular lesion and the BMD measurement the anatomical site of the vascular lesion has been noted. As described in the introduction there are several explanations behind the connection of low bone mass and vascular disease. One of them suggest that the negative remodelling of the bone is induced by the decreased blood flow and the consequent lack of nutrients. BMD is usually measured in 3 different anatomical regions, the radial and femoral head and the lumbar spine. In our study cohort, patients presenting with lower limb symptoms were arranged into 2 groups based on the anatomical site of the arterial stenosis. Patients with aorto-iliac disease were assigned to one group and patients with infra inguinal disease to another. The blood supply of the lumbar spine and the femoral head derives from the aorto and iliac vessels.

III.4. The role of Complement component 3 and fetuin-A in the development and progression of vascular calcification.

III.4.1. Association of the extent of PAD to complement component 3 and 4

In a cross sectional study design, 103 patients with lower limb atherosclerosis have been recruited. The previously described study questionnaire, laboratory measurements, evaluation of atherosclerosis and calcification have all been registered for these patients.

III.4.2. Laboratory measurements

In addition to the standard laboratory parameters, serum concentrations of C3, C4 were measured by radial immunodiffusion method [146]. The following reagents were used: Anti-human C3 Complement IgG Fraction, Anti-human C4 Complement IgG Fraction (DiaSorin Inc. Stillwater, MN, USA) for measuring the serum levels of C3, C4, respectively. For calculating the standard values Human Serum Protein Calibrator (DAKO A/S, Glostrup, Denmark) was used referring to C3, C4.

III.5. The role of complement component 3, 4 and fetuin-A in the progression of atherosclerosis

In this case control study 246 consecutive patients with severe peripheral artery disease were recruited at the Department of Vascular Surgery of Semmelweis University, Budapest in 2009. Follow up for them was organized 3 years after their visit to the Outpatient Department.

Serum fetuin-A was determined by the standard radial immunodiffusion method previously described in our group. Five microliters of patient's serum diluted to 1:4 was applied in 11.5 ml of Litex agarose gel (Sigma). Serum samples (1:4 dilution) with known concentrations of fetuin-A served as standards. The incubation was done at room temperature for 48 h. We used two types of antibodies against fetuin-A as the protein is synthesized as a single chain and is rapidly converted to a dipeptide form following the cleavage of a connecting peptide. The commercially available product (anti-fetuin-A,

IgG fraction, Incstar, Cat. No. 81931, 13.7 mg/ml, in a final concentration of 84  $\mu$ l/11.5 ml gel) recognizes the dipeptide form. The other type of antibody binding to the newly synthesized single chain form of fetuin-A, was raised by immunizing a rabbit with recombinant human protein (final concentration of 568  $\mu$ l/11.5 ml gel) [140].

Follow up for our patients was organised after 3 years to the original visit of the Outpatient Department. Another patient questionnaire was completed again. Patients were asked prior to their appointment to bring their medical documentation from the past 3 years and medical history was thoroughly revised. All cardiovascular events and their nature were noted. Patients were considered having myocardial infarction with noted rise and/or fall of cardiac biomarkers, with at least one of the values being elevated and symptoms of myocardial ischemia, or new (or presumably new) significant ST-segment/T-wave changes, or left bundle branch block, or development of pathological Q waves on ECG, or new loss of viable myocardium, or regional wall motion abnormality by imaging, or identification of intracoronary thrombus by angiography or autopsy [147]. Any episode of neurological dysfunction caused by focal cerebral, spinal, or retinal infarction that persisted beyond 24 hours or resulting in death within 24 hours [148] was considered as stroke or stroke-related death. All strokes were confirmed with magnetic resonance imaging or computed tomography. Any patient undergoing further vascular intervention was including open surgery and endovascular repair considered as a novel vascular event (NVE).

#### III.6. Statistical analysis

Statistical analysis was performed with Prism for Windows 5.01 (GraphPad Software, San Diego, CA) and SPSS for Windows 15.0.1 (SPSS Inc., Chicago, IL) statistical software products. As many of the variables had non-Gaussian distributions we used nonparametric tests in the analysis. We used the Mann-Whitney's U test to compare two independent groups, Kruskal–Wallis to compare multiple groups and Spearman's rho to calculate correlations. Multiple logistic regression analysis was also performed. All statistical analyses were performed two-tailed and p<0.05 was considered as significant. Values presented in the text are medians (interquartile ranges, /IQR/), unless otherwise

stated. For the univariate regression models we used Cox regression analysis or logistic regression

We were using the definition for Peripheral arterial disease (PAD) described by the National Institute of Health of the United States of America. We included all patients with clinically significant atherosclerosis on any site excluding the heart.

The Semmelweis University Regional and Institutional Committee of Sciences and Research Ethics approved the study protocol.

# **IV. RESULTS**

IV.1. Osteoporosis in the Hungarian population of patients with atherosclerosis

According to our knowledge, this is the first study investigating osteoporosis amongst patients with lower limb atherosclerosis. Table 3. presents the general clinical and biochemical characteristics of our 172 patients: 48 females and 124 males.

Table 3. Patient characteristics. Values are in median and interquartile range. Modified after Fehervari et al. [145]

	Patients
Number (n)	172
Age (years)	64(57-70)
Gender (male)	124(72%)
Smoking (n)	94 (55%)
Diabetes (n)	60 (35%)
Hypertension (n)	136 (79%)
Iliac disease (n)	76 (44%)
Infrainguinal disease (n)	96 (56%)
Dyslipidemia (n)	112 (65%)
Previously diagnosed osteoporosis (n)	16 (9%)
BMI (kg/m <sup>2</sup> )	25.98 (23.87/29.36)
Vitamin D3 (ng/mL)	21.25 (16.7/27.7)
Triglyceride (mmol/l)	1.70 (1.3/2.5)
Cholesterol (mmol/l)	5.20 (4.4/6.3)
LDL (mmol/l)	3.16 (2.6/4.0)
HDL (mmol/l)	1.37 (1.1/1.5)
Homocysteine (µmol/l)	17.0 (13.7/20.9)
Creatinine (µmol/l)	91.0 (81/105)
CRP	3.1 (1.0-7.1)
HBA1C	7.4 (6.6-8.4)

#### IV.1.1. Bone Mineral Density

As previously described, BMD was measured at 3 anatomical sites with DEXA scan. The median of the radial BMD was 0.86 (0.7-0.4) the femoral BMD was 0.83 (0.7-0.9) and the lumbar BMD 1 (0.9-1.2). We are displaying the median BMD, Z and T score values in different patient groups in Table 3. As described earlier, we identified important patient groups based on the risk factors of both conditions. The value of BMD on different anatomical sites for patients with different sex, age, BMI, smoking habits, are presented in Table 4. We compared these groups to each other, the level of significances also presented in the same Table.

	$1-BMD (g/cm^2)$	$f-BMD(g/cm^2)$	r-BMD $(g/cm^2)$
All patients	1.071	0.831	0.860
	(0.96 - 1.22)	(0.72 - 0.92)	(0.76 - 0.96)
Male	1.097	0.859	0.932
	(0.99 - 1.23)	(0.78 - 0.96)	(0.82 - 0.98)
Female	0.932	0.724	0.758
	(0.82 - 0.98)	(0.65 - 0.86)	(0.64089)
p value	0.109	0.007	0.004
Age < 64	1.103	0.859	0.904
	(0.98 – 1.28)	(0.73 – 1.00)	(0.82 – 0.97)
Age > 64	1.044	0.813	0.826
	(0.931.18)	(0.670.86)	(0.72 – 0.96)
p value	0.262	0.066	0.200
Obese	1.127	0.834	0.950
	(1.01 – 1.40)	(0.73 – 0.92)	(0.82 – 0.99)
Normal BMI	1.016	0.811	0.834
	(0.93 – 1.16)	(0.68 - 0.92)	(0.75 – 0.90)
p value	0.037	0.576	0.026
Non smokers	1.097	0.823	0.905
	(1.00 – 1.23)	(0.74 – 0.88)	(0.82 – 0.97)
Smokers	1.050	0.842	0.849
	(0.93 -1.22)	(0.68 - 0.93)	(0.74 – 0.96)
p value	0.421	0.689	0.203
Ex-smoker	1.095	0.831	0.877
	(1.00-1.31)	(0.68-0.93)	(0.76-0.98)

Table 4. Bone mineral density in patients with different risk factors of atherosclerosis and osteoporosis. Median values and IQR.

#### IV.1.2. The risk factors of osteoporosis and atherosclerosis

#### IV.1.2.1. Prevalence and gender specific comparison

Based on the T-score calculated from the bone density 64 (37%) of our patients had osteopenia and 53 (31%) had osteoporosis. Only 16 (9%) patients out of our cohort had been diagnosed with osteoporosis prior to this study and none of our patients were treated for osteopenia (Figure 5). We have organized our patients into females and males in order to evaluate the distribution of pathological bone absorption in these groups. Out of our 44 female patients 26 (59%) suffered from osteoporosis and 10 (23%) had osteopenia. Exactly 23 (18%) of our male subjects were affected by osteoporosis and 70 (45%) of them suffered from osteopenia. Statistical comparison of these two patient groups revealed significantly more patients with osteoporosis (P=0.00001) amongst female subjects. However, osteopenia occurred significantly more often in men (p=0.0002) (Figure 6.).



The prevalence of osteoporosis before and after DEXA scan

Figure 5. Bar chart displaying the percentage of bone disease before and after DEXA scan in patients with Peripheral Artery Disease



The prevalence of bone disease in male and female patients

Figure 6. Bar chart displaying the proportion of bone disease in patients with PAD of different sex. There are significantly more female having osteoporosis while more men have osteopenia.

#### IV.1.2.2. Age

The median age of our study cohort was 64 (57–70) years. As osteoporosis often worsens with age, we therefore divided our patients into 2 different subgroups based on their age. The first group was compiled of patients younger than the median age, 64 years, while the second group included individuals older than that. Exactly 82 patients, 24 women and 58 men, were older than 64 years at the time of this study. There were 90 subjects who were 64 years old or younger. The results of the DEXA scans of these groups is displayed in Table 3. Amongst older patients there were 35 (28.7%) suffering

with osteoporosis and 40 (36.6%) with osteopenia. In younger patients, the prevalence of both diseases –osteoporosis and osteopenia- were 18 (16.2%) and 24 (26.6%). However, the difference amongst these subgroups of patients was not significant neither for osteoporosis (p=0.6671), nor for osteopenia (p=0.5312) (Figure 7.)



The prevalence of bone disease in different age groups

Figure 7. Bar chart displaying the proportion of bone disease in the different age groups. There is no difference in the prevalence of bone disease between younger and older patients.

#### IV.1.2.3. Body Mass Index

The median of the BMI was 25.9 (23–29). Patients with BMI higher than 25 were recruited in to the first group and lower than this into the second group. Nobody had a BMI falling into the underweight or cachectic category. We found 28 (27%) patients with osteoporosis out of the 103 patients falling into the first group. There were 39 (38%) patients with impaired BMD. Amongst the 69 in the normal body weight group

there were equal numbers of osteoporotic and osteopenic patients, exactly 25 (36%). There was no difference between the two groups neither in the prevalence of osteoporosis (p=0.2397) nor in osteopenia (p=0.9730).



The prevalence of bone disease in patient with different body habitus

Figure 8. Bar chart displaying the proportion of bone disease in patients with different body habitus. The distribution is similar in patients across these groups.

#### IV.1.2.4. Smoking

The present and past smoking habit of our study cohort has also been recorded on the initial demographic questionnaire. At the time of the study 94 (55%) of the 172 patients were current smokers, however 151 (88%) patients were smokers for longer than 10 years during their life time. Based on the smoking history we divided our patients into 3 groups: current smokers, ex-smokers with significant smoking history and non-smokers. To demonstrate the effect of smoking we evaluate the prevalence of bone disease for these groups. Amongst current smokers 37 (39.3%) patients suffered with osteoporosis and 30 (31.9%) with osteopenia. Ex-smokers had a lower occurrence of bone disease

respectively 9 (15.7%) and 26 (45.6%) patients. Amongst non-smokers there were 7 (33.3%) osteoporotic patients and 8 (38%) had impaired burn turn over. By using Kruskal Wallis test we compared the BMD of these subgroups measured at different anatomical sites. There was no significant difference in any of the BMD amongst these groups. The statistical values were p=0.5525 at the lumbar site p=0.8110 at the femoral site and p=0.2213 at the radial head. The prevalence of osteoporosis is not different between the non-smoker and current smoker group p=0.6671, nor between the exsmoker p=0.5728 and current smoker group. The prevalence of osteopenia is not different amongst these groups, in a similar fashion: p=0.5312, p=0.6352 (Figure 9.)



The prevalence of bone disease related to smoking

Figure 9. Bar chart displaying the proportion of bone disease in smokers, ex smokers and non-smokers

IV.1.3. Bone turnover markers

The value of Vitamin D3, BAP-, PTH-, BGLAP-, bCTx are presented in Table 5. We presented the values of these osteoporosis markers in the male and female patients separately. These were the only subgroups with a significant difference in osteoporotic bone disease.

	Normal value	All patients	Male	Female
D3-vitamin	23-60 ng/mL	21.2 (16-27)	21.9 (17-28)	19.2 (14-25)
bCTx	0-320 pg/mL	354.0 (237-	332.0 (231-	426.5 (242-
		510)	467)	585)
BGLAP	20-48 ng/mL	17.6 (12.6-	16.8 (12-21)	19.6 (12-27)
		22.6)		
РТН	10-65 pg/ml	44.5 (34-61)	44.0(33-63)	47.0(38-61)
BAP	20-200 U/L	66 (49-86)	60(46-80)	81.0(63-101)
Homocysteine	3.4-20.4	17.0(13-20)	17.2(14-21)	16.1(12-18)
	µmol/L			

Table 5. The median (IQR) of bone turnover markers in different patient groups

# IV.2. Connection between osteoporosis and atherosclerosis

In order to assess the different factors influencing the previously reported connection between atherosclerosis and BMD we investigated several group of patients based on the previously suggested hypotheses. These groups were based on the anatomical site of the atherosclerotic lesion, the level of Vitamin-D and the diagnosis of dyslipidaemia. We present the general clinical characteristics of our patient in Table 6.

	Low vitamin- D3	Normal vitamin-D3	P value	Dyslipidemia	Without dyslipidemia	P value	Infrainguinal disease	Iliac disease	P value
Number (n)	68	104		113	59		96	76	
Age (vears)	63.7 (57- 69)	64.3 (59- 70)	0.325	62.9 (57- 68)	67.5 (60- 71)	0.067	65.1 (59- 72)	62.8 (57- 68)	0.084
Gender (male)	47	81	0.214	83	45	0.717	66	62	0.077
Smokin g (n)	39	55	0.639	62	32	1.0	42	45	0.065
DM (n)	21	39	0.415	40	20	0.867	38	22	0.152
(u)	56	80	0.446	92	44	0.326	80	56	0.134
BMI (kg/m <sup>2</sup> )	25.8 (23- 28)	26.1 (24- 28)	0.443	25.7 (23- 28)	27.2 (23- 31)	0.244	26.6 (24- 29)	25.5 (23- 28)	0.052

Table 6. Patient characteristics of the different subgroups (median values and IQR or number of patients, Mann-Whitney U or Fisher's exact tests)

IV.2.1. Bone mineral density is associated to the severity of atherosclerosis if the site of the lesion is considered

As displayed above, there was no significant association amongst BMD (T- and Zscore) and BS, without taking note of the anatomical site of the atherosclerotic lesion. As explained in the methods section II/2.2.2, this is important in order to assess the blood flow to the named bones. We divided our patients with lower limb ischemia into 2 groups. Patient with iliac disease and consequent diminished blood flow to the lumbar spine and femoral head were one group. The other group was compiled of patients with infrainguinal disease. We have found significant association in patients with iliac disease between BS and 1-BMD (p = 0.038, r = -0.467) and f-BMD (p = 0.002, r = -0.642), but we were unable to establish correlation between BS and r-BMD (p = 0.233, r = -0.306). Linear regression analysis demonstrated the connection between f-BMD and BS being independent from sex, age, BMI, and smoking habits (p = 0.001, RR= -1.5, 95 % CI of RR - 2.35 to - 0.684). In the infrainguinal group we did not find any association between BS and 1-BMD (p = 0.214, r = 0.118), BS and f-BMD (p = 0.505, r = 0.120) or BS and r-BMD (p = 0.202, r = -0.240).

# IV.2.2. BMD is not related to the severity of atherosclerosis in all patients

The angiography based Bollinger Score (BS) have been calculated for each of these patients in order to accurately describe the systemic blood flow. The median value of BS was 63.50 (37.00-84.00). The inter rater reliability for BS examiners was found to be a good agreement (kappa = 0.814).

The median BMD, T scores and Z scores and their Spearman correlation to BS are shown in Table 7.

	Median (IQR)	p and r value to Bollinger score
Bollinger score	63.5(37/84)	p=1
1-BMD	1.06( 0.96/1.19)	p=0.294 r=-0.147
l-T-score	-1.20 (-2.10/0.30)	p=0.302 r=-0.144
l-Z-score	-0.60 (-1.40/0.80)	p=0.118 r=-0.217
f-BMD	0.83 ( 0.72/0.92)	p=0.169 r=-0.192
f-T-score	-1.50 ( -2.40/-0.70)	p=0.197 r=-0.180
f-Z-score	-0.478 ( -1.10/0.10)	p=0.067 r=-0.253
r-BMD	0.860 (0.76/0.96)	p=0.052 r=-0.285
r-T-score	-1.150 ( -1.80/-0.30)	p=0.214 r=-0.185
r-Z-score	-0.20 ( -1.20/0.40)	p=0.038 r=-0.304

Table 7. There is no association between BMD and BS in all patients

To further evaluate this association between bone density and the lack of collateral circulation we compared the measured bone turnover markers to BMD in these important sites. We present these findings in Table 8. below.

	All patie	ents		Patients	Patients with iliac disea		Patients infraing	uinal dise	with ease
	l-BMD	f-BMD	r-BMD	l-BMD	f-BMD	r-BMD	l-BMD	f-BMD	r-BMD
bCTx	p=0.658	p=0.397	p=0.169	p=0.857	p=0.169	p=0.113	p=0.578	p=0.089	p=0.389
	r=-0.056	r=-0.107	r=-0.183	r=-0.038	r=0.284	r=-0.347	r=-0.091	r=-0.272	r=-0.148
BAP	p=0.279	p=0.475	p=0.421	p=0.378	p=0.451	p=0.277	p=0.669	p=0.019	p=0.825
	r=-0.136	r=0.090	r=-0.108	r=-0.184	r=-0.158	r=-0.243	r=-0.070	r=-0.369	r=0.038
PTH	p=0.918	p=0.068	p=0.396	p=0.178	p=0.178	p=0.154	p=0.514	p=0.349	p=0.787
	r=-0.013	r=-0.230	r=-0.115	r=-0.278	r=-0.278	r=-0.314	r=0.108	r=-0.154	r=-0.047
BGLAP	p=0.688	p=0.223	p=0.133	p=0.680	p=0.522	p=0.270	p=0.900	p=0.091	p=0.441
	r=-0.051	r=-0.153	r=-0.200	r=-0.087	r=0.134	r=-0.246	r=-0.020	r=-0.271	r=-0.132

Table 8. Burn turnover markers and their relations to BMD in different patient groups

IV.2.3. The role of Vitamin-D

In order to assess the effect of Vitamin-D on the connection between BMD and atherosclerosis we divided our study population into 2 subgroups. The general clinical characteristics and their distribution across these groups are displayed in Table 5. Patients having a serum concentration less than 20 ng/mL were considered in the low Vitamin D3 group [141], while the rest of the patients were allocated to the other group. In the low vitamin-D3 group we found that BMD was not associated to BS at any anatomical sites respectively BS to 1-BMD (p = 0.668, r = -0.108) BS to f-BMD (p = 0.990, r = 0.003), and BS to r-BMD (p = 0.087, r = -0.442). In a similar fashion in patients with normal vitamin D3, BS did not correlate significantly with 1-BMD (p = 0.465, r = -0.128), r-BMD (p = 0.355, r = -0.172). However, at the femoral site BS was

associated with f-BMD (p = 0.046, r = -0.340). We also compared the vitamin D3 levels in the subgroups created on the anatomical site of the atherosclerotic lesion (supra or infrainguinal stenosis) but we cannot report any association (p = 0.771) between these subgroups.

## IV.2.4. The role of Dyslipidemia

Dyslipidemia is an important factor for both calcifying diseases and has been highlighted as a potential link between them. The general clinical characteristics and their distribution across these groups are displayed in Table 5. Our results show no association in either of these groups between atherosclerosis and bone disease. The value of p and the regression coefficient in patients with abnormal lipid homeostasis were the following: BS to 1-BMD (p = 0.457, r = -0.121), BS to f-BMD (p = 0.169, r = -0.192), and BS to r-BMD (p = 0.052, r = -0.285). In patients without dyslipidemia: BS to 1-BMD (p = 0.457, r = -0.121), BS to f-BMD (p = 0.378), and BS to r-BMD (p = 0.223, r = -0.208).

# IV.3. The role of Complement component 3 and 4 in vascular calcification

Our study population consisted of 103 patients, 74 (71.8%) men and 29 women (28.1%) with symptomatic lower limb atherosclerosis and 109 healthy patients as the control. The past medical history for these groups is recorded in Table 8.

Table	8.	Clinical	characteristics	of	patients	and	controls.	Values	are	median
(interq	uart	ile range/	[QR/) or number	: (%	)					

	Patients	Control
Number	103	109
Age (years)	63 (55-69)	45(37-50)
Gender (male)	72%	49%
BMI	25.9(23.9-29.4)	25.6(23.0-28.4)
Smoking	59%	16%
Diabetes	38%	2,7%
Hypertension	74%	8,25%
Fontaine stages I, II/a, II/b, III, IV	0,9,66,17,11	109,0,0,0,0
C3 (g/L)	1.3(1.08-1.58)	1.08 (0.95-1.37)
C4 (g/L)	0.33(0.27-0.43)	0.35 (0.22-0.49)
Triglyceride (mmol/l)	1.6(1.1-2.2)	1.2 (0.8-1.7)
Cholesterol (mmol/l)	5.2(4.3-6.2)	5.4 (4.5-6.0)
CRP (mg/l)	4.2(1.1-9.4)	1.9 (1.2-4.5)
ABI	0.40(0-0.58)	
IMT mean (mm)	0.80(0.66-0.93)	
IMT maximum (mm)	1(0.80-1.3)	
Calcification score	5(3-6)	
Bollinger score	46(30-82)	
Insulin (pmol/l)	12.7(7.6-19.3)	
Hbalc (%)	7.4(6.5-8.2)	
C-peptide (ng/mL)	2.5(2-3.7)	

### IV.3.1. C3 is significantly higher in atherosclerosis than in healthy controls

In the atherosclerotic group the median of C3 was 1.36 g/l (IQR: 1.08-1.56) and it was 1.08 g/l (IQR: 0.95-1.37) amongst healthy individuals. Serum level of C3 was significantly higher in the lower extremity atherosclerosis group than in healthy subjects (p=0.00004). Please see Figure 10. The median of C4 was 0.33 g/L (0.27-0.43) in vascular patients and 0.35 g/l (0.22-0.49) in the other group. The level of C4 was not significantly different between patients with lower limb atherosclerosis and healthy controls (p=0.909) (Figure 11.).





Figure 10. Scatter plot diagram displays the connection between complement component 3 (C3) and healthy control. Mann Whitney U test.



Complement 4 in patients with lower limb atherosclerosis and healthy controls

Figure 11. Scatter plot diagram displays the connection between complement component 4 (C4) and healthy control. Mann Whitney U test.

#### IV.3.2. Clinical parameters and complements

We compared the results of each calcification assessment to both measured Complement Component in order to assess their relation to calcification. The median Ankle Brachial Doppler index was 0.4 (0-0.5). Its level was inversely associated with the level of C3 (r=-0.246, p=0.014) and C4 (r=-0.259, p=0.011) (Figure 12.). Out of the 103 patients, 38 required new angiography for clinical reasons. The Bollinger score based on these images was determined. We report an inverse connection between BS and the level of C3 (r= -0.357, p=0.028) (Figure 13.) but not to the level of C4 (r= -0.297, p=0.079) in this study cohort.

# Association of C3,C4 and ankle-brachial Doppler index



Figure 12. Scatter plot diagram displays the connection between complement component 3 (C3), complement component 4 (C4), and ankle-brachial Doppler index (ABI). Spearmen rank correlation [78].



Figure 13. Scatter plot diagram displays the association between complement component 3 (C3) and Bollinger angiographic score (n=38). Spearmen rank correlation, (p=0.028, r=-0.357)[78].

We evaluated our patients Fontaine stadium in order to compare them to the level of complements. Nobody in our study cohort had subclinical atherosclerosis (Fontaine I). A small number of patients, 8%, had clinically significant atherosclerosis but without the need for surgery (Fontaine II/a). The majority of our patients, 92%, needed surgical or endovascular intervention (Fontaine II/b-IV). By using Kruskall-Wallis' test we compared the different groups of patients based on their Fontaine stages. We cannot report a significant difference in the level of C3 (p=0.436) amongst these groups. Subgroups were created to assess clinical important features and the relation of C3. These groups have been compared to each other by Mann-Whitney's U tests. No significant difference can be observed amongst Fontaine group II/a and II/b (p=0.076) (need for surgery). The C3 level of patients in Group II and critically ischemic groups (Fontaine III-IV) was also not significant (p=0.812).

#### IV.3.3. Calcification and complements

The median of arterial calcification score was high (5), which corresponds with the severity of calcification in our patients. Comparison of the patients with Spearman correlation did not establish connection between CS and the level of C3 (r=-0.046, p=0.672) or C4 (r=-0.050, p=0.654). The levels of C3 and C4 neither correlated with the mean IMT (r=0.003, p=0.978), (r=0.039, p=0.736) nor with the maximum IMT (r=0.104, p=0.351), (r=0.083, p=0.468).

#### IV.3.4. Other findings

As expected, we found significant positive correlation between C3 and C-reactive protein (r=0.230, p=0.001). Many of our patients (38 %) were diabetic. Significant positive correlation was found between C3 levels and Hba1c (r=0.311, p=0.002), insulin (r=0.242, p=0.016) and Peptide-C (r=0.243, p=0.016). There were no correlations between C3 levels and triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL).

IV.4. The role of complement component 3, 4 and fetuin-A in the progression of atherosclerosis

IV.4.1. Complement 3 and 4 and future cardiovascular complication

The median age of our patient in this part of the study was 65 (57-71) years and the median BMI was 26 (23-29) kg/m<sup>2</sup>. More than 71% of our patients were men (n=177), 35% were diabetic (n=87) and 50% (n=123) were current smokers. However, more than 82% (n=202) of our patients smoked for a period longer than 10 years during their lives. There were relatively less smokers amongst the diabetic patients 41% (n=36). The median follow up time was 2.7 (2.6-2.8) years and 9.7 % of the patients died during this time (n=24). The mortality of diabetic patients was 9.1 % during the follow up. The median C3 was 1.31 (1.1-1.5) g/l. Patients were divided in to 3 groups based on any new cardiovascular events. The 3 groups were myocardial infarction (MI) (n=26), cardio embolic stroke (CES)(n=13) and novel vascular event (NVE)(n=54). We compared the baseline C3 of these groups by Mann-Whitney U test and found significant association amongst high C3 and MI (p=0.039) and NVE (p=0.011). Cox univariate regression estimated the predictive value of increased C3 for future MI (p=0.0001, HR: 1.56). We did not find significant regression between novel vascular events (p=0.06, HR:1.26) and baseline C3 or CES (p=0.14, HR:0.59) and C3. Table 2. depicts some of the other relevant parameters as predictors of the different cardiovascular events. To more accurately identify the group of patients having a higher risk for MI, we used ROC analysis, which suggested that patients having higher C3 than 1.55 g/L should fall in to this group. We displayed the ROC curves on Figure 14. Based on the ROC analysis we created 2 patient groups, patients having higher C3 than 1.55 g/l were considered as a high risk group. We compared high and low risk patients' survival with Kaplan Meier analysis. Please see the Kaplan Meier curves on Figure 15. The comparison of the two survival curves with Log-rank test revealed a significant difference of survival time (p=0.0001), with patients with a lower baseline C3 having a longer survival time. Neither Mann Whitney U test (p=0.499), nor Cox regression analyses reveal a connection between baseline C3 and cardiovascular mortality (p=0.99, HR:0.99).



Figure 14. ROC analysis of C3



Figure 15. Kaplan Meier survival analysis of patients with high and low C3

#### IV.4.2. Fetuin-A and other markers

We report the median and interquartile values of inflammatory parameters and some of the Framingham risk factors within the different groups in Table 2. Fetuin-A is a well-known parameter of calcification. The fetuin-A level of patients who died during the follow up was similar to survivals (p=0.839). In addition, the overall morbidity was not determined by its level (p=0.631). The comparison of the following patient groups to fetuin-A did not show significant association, respectively MI (p=0.863), CES (p=0.594) and NVE (p=0.339). Amongst the study cohort the level of C3 was associated to CRP (p=0.005), triglyceride (0.008) and Hba1C (p=0.002) but did not correlate with homocysteine (p=0.622), LDL (p=0.440), HDL (p=0.075), total cholesterol (p=0.308) or creatinine (p=0.602).

Patients who died during the follow up had higher BMI (p=0.012) but they were not significantly older (p=0.056), nor did their other baseline characteristics differ significantly from the patients alive at 3 years; respectively, gender (p=0.815), smoking (p=0.511), and diabetes (p=0.836). There was also no difference found between the level of CRP (p=0.359), Triglyceride (p=0.158), HbA1c (p=0.369), Homocysteine (p=0.194), HDL (p=0.565), LDL (p=0.562), and total cholesterol (p=0.368) within these groups.

### IV.4.3. Regression analysis

By using multivariate logistic regression, we adjusted some of these parameters to our previously described associations. We included Homocysteine, CRP, and Creatinine in our model and found that the association between baseline measurements of C3 and a

new onset of MI remained true after adjusting with these parameters. Please see the results displayed in Table 10.

Table 10. Multivariable prediction analysis using baseline variables

Hazard ratios refer for 1 standard deviation increase. In Model 2 hazard ratio of C3 > 1.55 g/l refers for presence versus absence. HR = hazard ratio, CI = confidence interval,  $\chi$ 2= Wald chi squared, CRP = C reactive protein, C3 = complement compound 3.

	Novel AMI (n=24)						
	HR	95% CI	$\chi^2$	р			
Model 1							
Creatinine (µmol/l)	1.17	0.82-1.66	0.81	0.36			
Homocysteine (µmol/l)	1.41	0.98-2.04	3.56	0.05			
CRP (mg/l)	1.25	0.99-1.57	3.65	0.05			
C3 (g/l)	1.59	1.11-2.27	6.54	0.01			
Model 2							
Creatinine (µmol/l)	1.14	0.77-1.69	0.47	0.49			
Homocysteine (µmol/l)	1.39	0.95-2.01	2.99	0.08			
CRP (mg/l)	1.26	0.99-1.60	3.79	0.05			
C3 > 1.55 g/l	3.96	1.69-9.28	10.06	0.002			

### V. DISCUSSION

## V.1. OSTEOPOROSIS AND ATHEROSCLEROSIS

#### V.1.1. General clinical and laboratory results

The basic demographic and clinical parameter of our atherosclerotic cohort is displayed in Table 2. As expected, we had more male patients than female. It is well known that atherosclerosis affects male patients more than females, therefore a group of consecutive patients will be likely to include more males. The high median age, and the high number of smokers, diabetics and patients with hypertension, as well as the lipid profile and homocysteine levels are all typical values for a cross section of atherosclerotic patients. The median level of CRP, however, is lower than it has been previously reported before and within the normal range. However, the upper quartile of CRP was higher than the normal values. The evaluation of dyslipidemia and Vitamin D3 is an important feature of this study as it has been highlighted as a possible link between osteoporosis and atherosclerosis. The number of patients with iliac and infrainguinal atherosclerotic disease has been displayed. All of our patients had lower limb symptoms and the site of the atherosclerotic lesion was either of these. We evaluated patients with existing diagnoses of osteoporosis at their first visit to our department. We found a low number of osteoporotic patient in this age group, about 20% of men and over 40 % of women were anticipated to have osteoporosis over the age of 50 [149] Furthermore, about 40% of these patients will suffer a fragility fracture.

BMI is an important factor in both diseases. In bone degenerative disorders high BMI appears to have a protective effect, whilst in atherosclerosis it is associated with worsened outcomes. Therefore, it was important to record precise BMI measurements including abdominal girth measurement, during the initial examination for this study.

### V.1.2. The prevalence of osteoporosis

The bone mineral density is the gold standard investigation to assess the presence of osteoporosis. It represents a relative value compared to the density of healthy bone. It was unexpected to see such a high number of osteoporotic patients in our cohort with lower limb ischemia. Females were about 20% more affected then the general age matched population, whist males were similar to it. However, the prevalence of osteopenia was unexpectedly high amongst them. This could suggest that these patients are losing their bone mass with a higher frequency than subjects not suffering from vascular disease. It is also important that only 9% of the patients were previously diagnosed with osteoporosis. There are several reasons that can be behind this. Patients with vascular disease are often less mobile and have multiple severe co-morbidities. One of the possibilities is that these patients simply do not attend to osteoporosis screening as they are occupied with much more severe problems. Other reasons can be that GPs are more focused on keeping these conditions under control and they miss sending these patients to DEXA scans. We would like to highlight the importance of screening and preventive treatment of osteoporosis in this population.

V.1.3. BMD and the mutual risk factors of bone disease and atherosclerosis

In Table 2. we display BMD values of some of our cohort. It is important to highlight that these results are displayed for descriptive purposes and to validate our study against previous findings. The prevalence of osteoporosis was significantly higher in female subjects while the prevalence of osteopenia was significantly higher in men. These findings are presented in Figure 2. Furthermore, as displayed in Table 2 we found a significant difference between male and female subjects in two of the measured sites of BMD. However, we were surprised to see how many male patients are affected by

pathologically decreased bone mass. The finding that significantly more male patients suffered from osteopenia suggests that both sexes are affected in this patient group. It is well known that the overall highest life time bone mass is lower in women and it stops increasing and begins to decrease at a younger age than in men. This results in a higher prevalence of osteoporosis. This is true in our atherosclerotic patients too. The median age of our study population was 64 years. Studies about osteoporosis often use 50 years of age as a point for subgroup analysis. It appears to be that this is the age when the decline of bone mass manifests in osteoporosis in many people. Bone mass, even under physiological circumstances, decreases by ageing. It has only been possible so far to slow down the process and avoid fragility fractures. Atherosclerosis presents at an older age on a larger scale. In our cohort it would not have been meaningful to divide our patients in to two groups by choosing 50 years of age as an analysis point as most of our patients were older than that. We chose the median age instead and arranged patients based on that. We cannot report a significant difference of the bone mass amongst these two groups despite ageing being a well-known risk factor for both diseases. The significant finding in femoral T-score is possibly a chance finding.

As highlighted earlier, BMI has a predictive role in bone disease, whilst it acts as a risk factor in atherosclerosis. The comparison of overweight and normal body weight patients has demonstrated significant differences in bone density. This was expected and confirms the protective role of obesity towards osteoporosis in patents with atherosclerosis. However, the number of osteoporotic and osteopenic patients was not significantly higher for patients with normal body habitus. This is probably due to an insufficient number of subjects. The significant difference in bone mass must manifest with a higher number of investigated patients based on the findings of BMD and BMI.

Despite being a major risk factor for both diseases, smoking does not seem to be influencing bone density in patients with chronic lower limb ischemia. We have conducted a thorough analysis as our initial thought was that smoking does influence BMD in atherosclerotic patients. In our study cohort we cannot describe a role for smoking. Further studies with different patient groups is needed to clarify the exact role of tobacco use.

V.1.4. Bone turnover markers and atherosclerosis

We investigated several markers of bone health in this study. We presented the values in Table 5. According to our knowledge, this was the first study in atherosclerotic patients investigating these important parameters. After the findings of increased osteoporosis, it was expected to find low levels of Vitamin D3. All the other values were within the normal limits. Less is known about the effect of atherosclerosis in these parameters. We compared the value of these markers between female and male subjects as this were the only subgroup where we were able to demonstrate a difference in BMD. We cannot report any significant associations. The level of homocysteine was on the higher fields of the normal values and the upper quartile of our patients were outside this range. This is a biochemical evidence of atherosclerosis in our cohort.

### V.2. What is the origin of the connection of atherosclerosis and low bone density?

The general medical parameters of the subgroups we compared to investigate the effect of Vitamin D3, dyslipidemia and the site of the atherosclerotic lesion are presented in Table 6. These are the original subgroups identified prior to our investigation as being of interest based on previous findings. As demonstrated, there are no differences between these groups in any of the general medical parameters.

### V.2.1. About the blood supply of the sites of the BMD measurements

Bone mineral density in our research was measured at 3 anatomical sites: radial and femoral head and lumbar vertebrae.

The blood supply of the femoral head and the lumbar spine derives from the aorto-iliac vessels. The majority of the blood supply of the femoral head derives from the ascending branch of the lateral circumflex femoral artery. This is a branch of the profound femoral artery in 60% of the cases, in other instances it derives from the superficial or common femoral artery. This branch anastomoses with the deep iliac circumflex artery. If the profound femoral artery is obstructed this vessel will provide the blood supply to the femoral head. The blood supply of the trochanters and the neck of the femur derives from the ascending branch of the medial femoral circumflex artery,

which is again usually a branch of the deep femoral artery [150]. The blood supply of the lumbar vertebrae is provided by the lumbar arteries, which all derive from the abdominal aorta. The blood supply of the radial head derives from the radial artery.

### V.2.2. Site specific comparison

Based on the site of the arterial stenosis, we divided our patients into patients with supra-inguinal stenosis and infra-inguinal stenosis. It was not possible to recruit a sufficient number of patients having purely distal stenosis with no atherosclerotic lesion on the femoral arteries. However, as described above, there was no need to do this as in the case of a profundal or common femoral lesion, the blood flow in the deep circumflex arteries will provide the supplements to the femoral head through the direct anastomosis in between these vessels. Our idea was that patients with reduced blood flow to the femoral head and lumbar vertebrae should have lower bone density than patients without aorto-iliac lesions. Furthermore, the BMD measured at the radial head should be higher than on the other two sites. To describe the reduced blood flow, we used a less known and slightly more complicated Bollinger score system. The advantage of this scoring is that it precisely describes the collateral circulation of the relevant anatomical area. The disadvantage is that the scoring is difficult and requires experience and time to evaluate it. As described in the results, we found a significant negative correlation between BS and lumbar and femoral BMD in patients with aorto-iliac disease. As expected, radial head bone density was not associated to it. The same association was not true in patients with infrainguinal disease on any of the anatomical site. This supports our hypothesis regarding the origin of decreased bone density in atherosclerotic patients.

The majority of our patients, as expected in an atherosclerotic cohort, were male. As highlighted before, female sex is a risk factor of osteoporosis. Age and smoking is a risk factor of both diseases while BMI seems to have an opposite role in each disease. Therefore, there was a clear need to evaluate the importance of these factors. Our linear regression model compiled of these factors demonstrated that the connection described above is independent of these factors. All of these findings, but especially the ones

regarding BMI and sex, highlight their importance as they suggested atherosclerosis is an independent risk factor of osteoporosis in our study cohort.

# V.2.3. General findings

All of our patients in this study had ischemic lower leg symptoms.

General comparison of Bollinger score to BMD did not reveal any significant association as presented in Table 5. Initially, it was difficult to understand why the severity of atherosclerosis is not related to BMD. However, by understanding the importance of the blood supply to each bony area clarifies this issue.

# V.2.4. The role of Vitamin D

As detailed in the introduction, vitamin D is an important marker in both diseases. Previous research highlighted that this vitamin can be responsible for the decreased bone mass in vascular patients. We divided our patients in to two groups based on their level of vitamin D. In the case of hypo-vitaminosis bone density should be decreased. However, our findings cannot confirm this but we do not suggest to reject this hypothesis. Our measurements were carried out on one occasion only and during the winter period. It is well known that the active form of vitamin D can only be synthetized in humans if they are exposed to a sufficient amount of sunlight. Therefore, it is possible that many of our patients did not suffer from chronic hypovitaminosis D, but instead were not exposed to enough sunlight at the time of their blood samples. Increasing amount of evidence suggests important role of vitamin D not only in bone disease, but also in atherosclerosis and several other diseases such as depression or skin conditions. We think it is important to diagnose hypovitaminosis D in the maximum number of patients as early as possible and treat them appropriately to avoid future significant morbidity.

V.2.5. Dyslipidemia
The importance of abnormal lipid homeostasis does not need to be emphasized. Unlike previous authors, we did not find any relevance of dyslipidemia in the connection of bone and vascular disease. Some of the previously cited studies, however, did suggest a role. It is possible that clear associations cannot be determined in our study cohort as many of our patients with dyslipidemia were on treatment at the time of the study, which could have interfered with our results. Solely analysing data from patients with impaired lipid metabolism without receiving anticholesterol therapy would have been biased as it was not clearly identified why some of the patients were receiving lipid lower therapy whilst others were not. Dyslipidemia is probably the hypothesis most difficult to assess in this patient group. It is not possible in modern societies to find a group of randomly selected atherosclerotic patients without anticholesterol treatment. A possible way of evaluating the role of lipids could be multiple longitudinal measurements and comparison of both BMD and lipid profile.

### V.2.6. Bone turnover markers

The importance of burn turnover markers has been explained. In our study we evaluated their levels in relation to BMD in order to find an association between biochemical markers of osteoporosis and atherosclerosis. As displayed in Table 7 we could not find any relevant association between these parameters, neither in all the patients nor by following the previously described pattern. This could be a result of many things. The most important point is to highlight that the level of these factors changes with "traditional" osteoporosis. The fact that we cannot describe significant association relating to these turnover markers could support the hypothesis that bone disease is based on different mechanisms – such as deprived blood flow- in atherosclerosis.

#### V.3. The role of complement component 3 and 4 in the progression of atherosclerosis

#### V.3.1. Patient characteristics

As described earlier complement components have an important role in vascular calcification. C3 is the central component of the complement system, whereby the

molecule activates the terminal pathway, while C4 is the key member of the classical and mannose-biding pathway. These pathways are most likely to be relevant in calcification [77]. For this study we investigated patients with clinical symptoms of lower limb ischemia. In table 8 we display the baseline clinical and laboratory characteristics of our study population. The findings presented in the first column resemble a typical picture of groups of patients suffering with vascular calcification. Important values of our control groups are displayed in column two. They display a typical cross section of the population. As vascular calcification typically manifests as clinical symptoms later on in life it has not been possible to find an age matching healthy control group.

## V.3.2. Level of Complements in patients and controls

Comparison of patients with lower limb ischemia to a healthy control suggested a significant increase in the level of C3. This has been previously described by Muscari et al [151] and we only present it here to validate our study against known international standards. The level of C4 however was not significantly higher in patients with atherosclerosis. Previous studies reported C4 being elevated in atherosclerosis, however population based studies could not confirm this [77]. Furthermore, it has been reported previously that C4 has an association with the life span in the Hungarian population via the decrease expression of C4B allele by ageing [152]. As the level of C4 increased in patients with atherosclerosis and decreased in elderly Hungarian patients it is difficult to compare our study cohort to healthy individuals. The purpose for the measurement of C4 was to compare its level to the markers of severity of atherosclerosis and calcification.

# V.3.3. Complement components and the clinical severity of atherosclerosis

V.3.3.1. ABI and Bollinger score associated to the level of complements

Ankle Brachial Doppler index is a simple, frequently used method for rapid assessment of circulation. All of our patients had clinical symptoms, which is represented in the low

median values of ABI. We found significant association between worsening ABI and the level of C3 and C4. This is an important finding because this has not been described before and it indicates that C3 and C4 could be used in the assessment of atherosclerosis and its risks. The finding regarding C4 confirms that our study is valid. We also compared complement components to Bollinger score. This revealed significant inverse correlation between C3 and BS but not between C4 and BS. However, the value of p for C4 was very close to 0.05. As previously described, only a limited number of patients, 38 had new angiography for clinical reasons as the rest of the patients were treated based on images taken in the previous 12 months. Therefore, it is possible that this association would have been significant if we had a larger number of patients to compare. The importance of these findings, besides identifying new factors that can be an aid in the assessment of atherosclerosis, is to more precisely estimate the severity of the disease. Furthermore, the measurement of complements is a less operator dependent method and does not require specific skills like ABI. Therefore, after further studies, these molecules might be a tool in population based screenings. The use of complements could help to reduce the number of patient visits and can be an effective tool to identify individuals in risk of atherosclerosis.

## V.3.3.2. Complements and the Fontaine stadiums

We compared the baseline level of complements in patients with different Fontaine stadiums. We cannot report a significant difference, neither in the level of C3 nor in the level of C4, amongst patients with different stadiums. Based on the previously described association amongst ABI and complements we could expect a difference. The most appealing finding in our results is between patients requiring and not requiring surgical intervention (Fontaine II/a and II/B). The difference amongst these groups could again be significant if we had a larger sample of patients to investigate. Differences between caludicants and patients with critical ischemia, however, may not be of a great interest as these clinical manifestations are multi factorial and often determined by the development of collateral circulation. This again relies upon many patient related factors such as exercise activity or diabetes.

#### V.3.4. Calcification and the complements

Complements are present and activated in the atherosclerotic plaque and have been identified as one of the factors responsible for plaque instability. The question, however, remains as to whether C3 and C4 could play a role in the development of calcification. To investigate this, we compared their level to several parameters describing calcification directly or indirectly. Our hypothesis was that more calcification should be associated with higher complement activity. For this purpose, we used an ultrasound based calcification score. In our patients we did not find higher C3 and C4 levels in patients with more extensive calcification. We also compared their level to carotid intima media thickness, partially to validate our study. It has been known for some time that C3 does not correlate with femoral or carotid IMT in healthy subjects but its relation to it was unknown in symptomatic patients [153]. Complement C4 also has not been evaluated in symptomatic patients yet. Our findings regarding IMT are very similar to the ones described in healthy individuals. The importance of CaxPo regarding calcification has been described. We cannot report its value being related to the level of complements. As expected, the well known association between complements and CRP was significant amongst our subjects too. The role of complements in diabetes has been highlighted several times [154]. In our study the level of C3 was associated to several diabetic markers such Hba1c. this suggests that the association between complements and diabetes is valid in patient with atherosclerosis too.

# V. 4. Complement and the progression of atherosclerosis

#### V. 4.1. Introduction

In this part of the study we evaluated the connection between baseline C3 levels and the progression of cardiovascular disease in patients with peripheral artery disease. It has been highlighted that elevated C3 is associated with early restenosis in carotid artery disease [155] and also influences the outcome of cardio embolic stroke, but has no effect on small vessel disease related cerebrovascular events [156]. However, other studies found no significant association between incidence of stroke and the level of C3

[88]. Our study population's smoking habits and the prevalence of diabetes are worse than previously reported in PAD patients [157]. Our observations based on a midterm follow up reported 9.7% mortality for all patient groups and 9.1% for diabetic. The mortality of the whole patient groups is worse than previously reported, however the mortality for diabetic patients is better [158]. This could be due to the high prevalence of smokers and ex-smokers [157], and fewer smokers amongst diabetic patients.

# V.4.2. Baseline C3 predicts future MI

We divided our study cohort based on their novel cardiovascular morbidity. There were no other problems considered in these groups than myocardial infarction, cardio embolic stroke and obliterative vascular disease. Patients with MI had a significantly higher baseline C3. This suggests that C3 can act as a risk factor for future cardiac events in patients with PAD. Based on this, the previously described association in healthy subjects can be valid in this patient group too. The estimated odds ratio suggests a moderate risk for worsening of atherosclerosis amongst patients with a high baseline C3. Regarding stroke, there are incongruent findings in the literature [88, 156]. As we had very low number of CES (n=13) compared to the size of our patient group (n=246), we could not possibly evaluate the connection between C3 and patients suffering from CES during the follow up. The predictive value of Framingham and other potential risk factors is presented in Table 2. As suggested in our introduction, these parameters not always reliable in judging the progression of atherosclerosis in symptomatic patients. ROC analysis suggested that the cut off point for high and low C3 should be 1.55 g/l in our patient cohort. Previous authors reported C3 higher than 1.8 g/l as pathological [159]. We divided our patients in to low and high C3 groups and compared the incidence of AMI. The Kaplan-Meier curves of these groups are displayed in Figure 11. The significant differences we found amongst the new onset of MI and C3 suggests that baseline C3 is a risk factor for future cardiac morbidity.

V.3. Complements, mortality and morbidity

In the present study, we also analysed the effect of a higher baseline C3 for cardiovascular and all cause mortality. We did not find a high C3 level being predictive for either of them. There was no significant difference amongst the baseline characteristics and atherosclerosis risk factors to the survival patients.

In our investigations we successfully proved however that a high baseline C3 predicts a new onset of myocardial infarction. Furthermore, patients suffering a new vascular complication has significantly higher C3. This means that C3 can be an aid to identifying vulnerable patients for rapid deterioration of atherosclerosis. It is also suitable to highlight patients with increased risk of myocardial infarction. The need of future vascular intervention could also be estimated based on the baseline level of C3.

### VI. CONCLUSION

Results of performed investigations - what did this study add to our current knowledge?

An increase prevalence of osteoporosis was found in patients with lower limb atherosclerosis. This study was the first analysing all 3 hypotheses in patients with symptoms of lower limb atherosclerosis. Our results suggest that the loss of bone mass is due to the reduce blood flow rather than low vitamin D3 or dyslipidaemia. The level of complement component 3 is associated to ankle brachial pressure index and angiographic Bollinger score in patients with chronic lower limb ischemia. Furthermore, the baseline level of complement component 3 independently determines future myocardial infarction in patients with peripheral artery disease. The important role of complements on calcification has been highlighted before, however less were available about it's relation to clinical parameters and clinical outcomes. Despite Fetuina is an indicator of the extent of calcification it does not determine the outcome of worsening atherosclerosis.

## VII. SUMMARY

The development of calcification and atherosclerosis are frequently investigated but there are many aspects of them that is still unknown. Recent studies highlighted a connection between atherosclerosis and bone density in healthy subjects. This is one of the first studies investigating this connection in patients with atherosclerosis. Also, the prevalence of osteoporosis has never been investigated in the Hungarian population of patients with peripheral vascular disease. Several hypotheses were presented previously to explain this connection, we further investigated them. Calcifying factors effecting the severity and the progression of atherosclerosis have also been evaluated in this study. In the dissertation after a thorough review of the literature regarding atherosclerosis and calcification it has been successfully demonstrated that osteoporosis often affects patients with atherosclerosis and thoroughly described the characteristics of this patient group. We demonstrated connection between bone mineral density and reduced collateral circulation. Out of the three existing hypotheses regarding the link between them we were able to demonstrate an important role to reduced blood flow but did not find Vitamin-D or dyslipidaemia important in our patient group. The role of complement components in atherosclerosis has been further clarified by describing them being associated to ankle brachial index and angiographic score. We were able to demonstrate that C3 is an independent risk factor for myocardial infarction in patients with atherosclerosis.

These findings can help to treat patients with atherosclerosis and to better evaluate their current and future cardio-vascular condition.

# VIII. ÖSSZEFOGLALÁS

A vaszkuláris kalcifikáció és az arterioszklerózis kialakulása egy gyakran kutatott tudományos téma, azonban továbbra is sok kérdés megválaszolatlan velük kapcsolatban. A közelmúltban több szerző is kapcsolatot írt le az érelmeszesedés és a csontsűrűség között egészséges Egyedekben. Kutatásunk az elsők között van amely perifériás érbetegek körében vizsgálja a csontsűrűséget és a csontritkulás előfordulását. A fent leírt kapcsolat magyarázatául több hipotézis is született melyeket részletesen megvizsgáltunk a vaszkuláris és extravaszkuláris kalcifikáció mértékért és az érbetegség rosszabbodásáért felelős paraméterekkel együtt.

A dolgozatunkban az irodalom részletes áttekintését követően bizonyítottuk hogy csontritkulás gyakrabban fordul elő érelmeszesedésben szenvedő betegekben. Három különböző elméletet azonosítottunk az irodalmi áttekintés során. Betegeink körében a csontok csökkent vérellátása bizonyult a fő tényezőnek amely az oszteoporózis kialakulásához vezetett míg az alacsony vitamin-D szint illetve a diszlipidémia nem bizonyult jelentős tényezőnek. A komplement fehérjék és az arterioszklerózis kapcsolatáról sikerül az eddig megismerteken túl bizonyítanunk, hogy egyes komplementek szintje összefügg a betegség klinikai vizsgálatokkal által leírt súlyoságával, azonban szintjük nem függ össze a kalcifikáció mértékével. Sikerült továbbá bizonyítanunk hogy a magas C3 szint előre jelzi a szívinfarktust érbetegek körében. Kutatásaink közvetlen módon segítik az érbetegségben szenvedők gyógyítását és segítséget nyújt a középtávú kardiovaszkuláris kockázat megítélésében.

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# Köszönetnyilvánítás

Köszönöm Acsády György professzor úrnak, aki témavezetőként, érsebész professzorként lehetővé tette számomra, hogy ez a kutatás illetve doktori disszertáció létrejöjjön. Tanácsai nagy segítségemre voltak a tudomány sokszor nehezen érthető világában.

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Doktori disszertációm nem jöhetett volna létre szüleim, feleségem és családom támogatása, szeretete és türelme nélkül.